



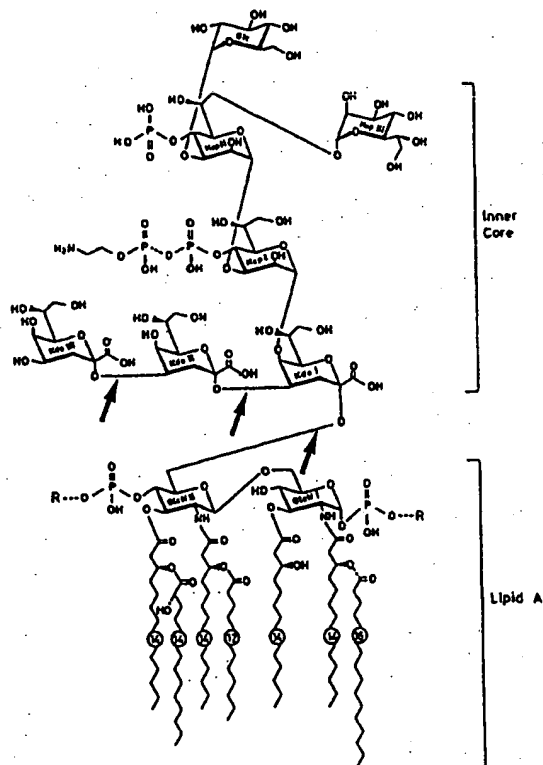
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : G01N 33/569, 33/543, C07H 3/04, 3/06		A1	(11) International Publication Number: WO 00/36419
			(43) International Publication Date: 22 June 2000 (22.06.00)
(21) International Application Number: PCT/DK99/00704		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (Utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 15 December 1999 (15.12.99)			
(30) Priority Data: PA 1998 01655 15 December 1998 (15.12.98) DK			
(71) Applicant (for all designated States except US): EXIQON A/S [DK/DK]; Bygstubben 9, DK-2950 Vedbæk (DK).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): JAKOBSEN, Mogens, Havsteen [DK/DK]; Alekistevej 225, DK-2720 Vanløse (DK). BOAS, Ulrik [DK/DK]; Prinsesse Charlottesgade 5, 4. th., DK-2200 Copenhagen N (DK). JAUHO, Eva, Irene, Stenbæk [DK/DK]; Højmossevej 34, DK-2400 Copenhagen NV (DK). HEEGAARD, Peter, M., H. [DK/DK]; Nordborggade 10, 2. th., DK-2100 Copenhagen Ø (DK).			
(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annæ Plads 11, P.O. Box 3007, DK-1250 Copenhagen K (DK).		Published With international search report.	

(54) Title: COUPLING OF LIPOPOLYSACCHARIDE-DERIVED CARBOHYDRATES ONTO SOLID SURFACES

## (57) Abstract

The present invention provides a method for immobilising a polysaccharide (PS) to a solid surface, said polysaccharide having a keto-carboxy group ( $-C(=O)-COOH$ ) or a ketal or hemiketal group corresponding thereto, e.g. derived from KDO (2-keto-3-deoxy-D-mannooctonic acid), the method comprising the steps of: (a) forming a covalent bond between the carboxy group of the polysaccharide and a reporter molecule (RM). Comprising a recognition/substrate site (e.g. biotin or an anthraquinone); and (b) immobilising for diagnostic purposes, e.g. for the detection of bacterial infections from Gram-negative bacteria.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## COUPLING OF LIPOPOLYSACCHARIDE-DERIVED CARBOHYDRATES ONTO SOLID SURFACES

### 5 FIELD OF INVENTION

The present invention relates to a method for immobilising special classes of polysaccharides to solid surfaces. Such a method is highly valuable in the construction of reliable assays for the detection of an antibody corresponding to the polysaccharide  
10 antigen. The present invention also relates to modified solid surfaces and to the use of such surfaces in various diagnostic assays. Furthermore, the present invention relates to novel KDO derivatives which are valuable intermediates in the construction of such modified solid surfaces.

### 15 BACKGROUND OF INVENTION

Bacterial lipopolysaccharides (LPSs) are characteristic outer membrane constituents of Gram-negative bacteria. LPSs are widely used as antigens in diagnostic assays specially designed for the specific detection of antibodies in serum, plasma, meat juice, saliva or  
20 other body fluids, originating from bacterial infections in humans and animals. LPSs are highly immunogenic and comprise one of the epitope characteristics for a given bacterial strain. In fact, the definition of a serotype is often based on the LPS and/or capsular polysaccharide (CPS) antigenicity. The antigenic specificity of the LPS molecule resides in the polysaccharide part of the LPS, the O-antigen, whereas the toxicity of the LPS is  
25 caused by residues contained in the lipid part of the LPS, called the lipid A. LPSs are highly amphiphilic compounds because of the joint presence of a hydrophilic O-polysaccharide group and a hydrophobic lipid group in the LPS molecule. Most of the characterised LPSs have the same principal structure which is especially conserved in the lipid A and in the inner core parts of the LPSs. The core is the part of the polysaccharide  
30 that comprises the bond between the O-antigen and the lipid A. This bond is invariably comprised of a ketosidic bond between the hemiketal function of the innermost KDO-residue and a hydroxyl-group of a GlcNAc-residue of the lipid A. The O-antigen of a specific bacterial serotype varies with respect to numbers of repeating units and may contain non-stoicheometrical substitutions with acetyl, phosphate, glycosyl or other groups.  
35 Generally, LPS-molecules without O-antigens, that is carrying only (parts of) the core

saccharides in addition to the lipid A are called "rough" LPS, while LPS-molecules carrying O-antigens are called "smooth" (Raetz, C. R. H. in *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (Neidhardt, F. C. E. A., ed.) Vol. 1, 2nd Ed., pp. 1035-1063, American Society for Microbiology, Washington D.C., 1996; Hitchcock et al, 1986, J. Bacteriol. 166, 699-705).

ELISA, enzyme-linked immunosorbent assay, is a well known method for detection of antibodies. In this assay, the LPSs are coated (or immobilised) on a solid surface (e.g. a plastic surface) by passive adsorption, where they serve as probes for specific antibodies.

10 The method consists of incubation of the LPS-coated surface with the biological sample being assayed for the presence of antibodies, followed by incubation of the LPS-antibody complex with a labelled secondary antibody.

Previously, LPSs have generally been immobilised onto a solid surface without any

15 modification of the molecules since the hydrophobic lipid A part of the molecules functions as a fairly efficient "anchor" binding the LPSs to the surface via non-covalent hydrophobic interactions leaving the hydrophilic O-polysaccharides pointing outward accessible for interactions with binding components, e.g. antibodies. However, it has been shown that the efficiency by which the LPSs are immobilised onto hydrophobic surfaces depends on

20 both the nature of the surface and the equilibrium between free LPSs and formed LPS micelles. The equilibrium between free LPSs and formed LPS micelles depends on the amphiphilic nature of the LPSs and varies between LPSs from different bacteria strains as well as between different LPS serotypes. Certain types of LPSs have shown to be very difficult to immobilise onto solid surfaces by non-covalent bonds without addition of

25 various micelle-dispersing agents (detergents) to the coating solution.

Thus, the optimal coating conditions vary among LPS from different bacteria strains as well as between serotypes of the same bacteria, making simultaneous immobilisation of two or more different LPSs onto the same surface very difficult. This is envisaged to be

30 due to the ability of a well-coating LPS type to compete out the less well-coating type. This phenomenon was illustrated with *Salmonella* Infantis LPS which was shown to coat inefficiently to plastic. In an assay for detection of *Salmonella* Typhimurium and *Salmonella* Infantis specific antibodies this lead to the substitution of *Salmonella* Infantis LPS with *Salmonella* Choleraesuis LPS which was found to coat much better and which carries the

35 same antigenic serotypes (O-antigens) (Nielsen, B. et al., 1995, Vet. Microbiol. 47, 205-218)

The tendency of LPS to form micelles (or aggregates) is furthermore believed to reduce the stability of the LPS coating as the interactions between bimolecular (or aggregated or cluster) LPS and the surface are believed to be weaker than the interactions between a  
5 single molecule of LPS and the surface. In other words, the potential tendency of LPS to coat in clusters may lead to a decreased and unpredictable coating stability and reduce the long-term stability of the coating.

EP 0 101 119 describes the immobilisation of a lipopolysaccharide to an insoluble carrier  
10 by the condensation of the lipopolysaccharide and either an amino or carboxyl group of the insoluble carrier.

JP-A-2-242448 (Patent Abstract of Japan) describes the immobilisation of a lipid A glycoside of 3-deoxy-D-manno-2-octurosonic acid to the surface of an insoluble carrier  
15 through an amide bond.

Highly hydrophilic antigens like e.g. bacterial polysaccharide (PS) are often very difficult to adsorb (immobilise) onto the most commonly used surfaces used in serological assays, such as plastics used in ELISA and RIA (radio-immuno assay), latex particles used in  
20 agglutination techniques and PVDF (polyvinylidenedifluoride) as well as nitrocellulose and other materials used for dip-stick, blotting or other fast assays. Accordingly, PS as such coat inefficiently and demand the use of large quantities of polysaccharide antigen or extremes of pH and can not be used with mixtures of polysaccharides (Elkins et al., 1990, J. Immunol. Meth. 130, 123-131). On the contrary, in spite of the drawbacks mentioned  
25 above LPSs are almost exclusively used as coating antigens as the lipid A provides the required hydrophobicity needed for adequate coating of the surface with antigen.

PSs have previously been isolated from LPSs with the purpose of preparing conjugates of PS and carrier substances, most often carrier proteins for vaccine purposes (Aron et al.,  
30 1993, J. Clin. Microbiol. 31, 975-978; Lambden and Heckels, 1982, J. Immunol. Meth. 48, 233-240; Gupta et al., 1995, Inf. Immun. 63, 2805-2810).

Previously, it has been shown that the polysaccharide part of *Salmonella* Typhimurium lipopolysaccharide (LPS) could be derivatised with biotin and immobilised onto a  
35 streptavidin-coated ELISA-plate where it could be recognised by antibodies against PS.

By using the hydrazide derivative of biotin, it was possible to react the PS directly with biotin-hydrazide without a prior oxidation step; the hydrazide was shown to react with the hemiketal of the reducing end KDO of the PS. This procedure while leading to antigenically intact biotinylated PS-derivatives, however resulted in derivatives that were not stable, and an avidin or streptavidin coating had to be introduced in the assay (Wiuff, C., Lind, P., Heegaard, P., 1997, Regioselective coupling of reducing carbohydrates to hydrazides for derivatization of bacterial polysaccharides and application to immunoassays, Proc. 2<sup>nd</sup>. Carbohydrate Engineering Meeting, La Rochelle, France, p. 66).

10 In Meikle et al. (Glycoconjugate J. 7, 207-218, 1990) a PS is used in an ELISA by directly coupling the PS to a detection enzyme in a competitive set-up. The coupling was performed by reductive amination of the keto functionality of the keto-carboxy group of a KDO unit of the PS. The PS/detection enzyme conjugate was not used for coating but was used in solution in a later step in the assay.

15 Generally, binding of PS to solid surfaces requires modifications of the PS molecule, but in such a way the O-antigen part stays unaltered. A number of such modifications have been described for naturally occurring polysaccharides. A well-known example is the coupling of capsular polysaccharides (CPSs), which do not contain hydrophobic parts (or groups), to proteins (Laferrière et al., 1997, Vaccine 15, 179-186; Beuvery et al., 1986, Develop. Biol. Standard. 63, 117-128.). The resulting capsular polysaccharide-protein complexes are then adsorbed to the surfaces through hydrophobic groups in the carrier protein. Bacterial capsular polysaccharides have also been modified by non-regioselective reaction of hydroxyl groups with hydrophobic groups e.g. phenyl or thyramine to enhance the overall hydrophobicity of the CPS molecules and thereby the binding abilities to solid surfaces (Kristensen and Bentzon, 1992, APMIS 100, 142-146).

A major problem is that antigenicity may be difficult to retain after derivatisation of the polysaccharide, as most methods are not directed to the derivatisation of any specific region of the polysaccharide antigen, thereby possibly destroying or modifying antigenic epitopes of the polysaccharide.

This has been overcome with the present invention by restricting the derivatisation to certain regions of the carbohydrate.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic molecular structure of *E. Coli* K12 LPS. Abbreviations: GlcN, D-glucosamine; Kdo, 3-deoxy-D-manno-octulosonic acid; Hep, L-glycero-D-manno-heptose;

- 5 Glc, D-glucose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Rha, L-rhamnose; Galf, D-galactofuranose; P, phosphate; P-Etn, phosphoethanolamine; P-P-Etn, ethanolamine pyrophosphate; Ac, acetate.

Figure 2: Shows the inner core and lipid A part of LPS from a typical Gram-negative  
10 bacteria.

Figure 3: UV spectroscopic profile of LPS from *Salmonella* Typhimurium.

Figure 4: UV spectroscopic profile of LPS from *Salmonella* Choleraesuis.

15

Figure 5: Left: Silver stained polyacrylamide gel of purified LPS from *Salmonella* Typhimurium. Lane 1: Low molecular weight marker (BioRad); 2: Empty; 3: 4 µg LPS; 4: 8 µg LPS; 5: 12 µg LPS; 6: 16 µg LPS; 7: 20 µg LPS; 8: 24 µg LPS; 9: Empty. Right: Coomassie stained polyacrylamide gel of LPS from *Salmonella* Typhimurium. Samples  
20 applied as above.

Figure 6: Left: Silver stained polyacrylamide gel of purified LPS from *Salmonella* Choleraesuis. Lane 1: Empty; 2: Low molecular weight marker (BioRad); 3: Empty; 4: 6 µg LPS; 5: 12 µg LPS; 6: 18 µg LPS; 7: 24 µg LPS; 8: 30 µg LPS; 9: 36 µg LPS; 10: Empty.  
25 Right: Coomassie stained polyacrylamide gel of LPS from *Salmonella* Choleraesuis. Samples applied as above.

Figure 7: Indirect ELISA of LPS and of PS from *Salmonella* Typhimurium. The antigens were coated in a 2-fold titration row starting from 0.4 µg/mL. A *Salmonella* Typhimurium positive  
30 swine serum diluted 1/400 was used as antibody. (Samples appear in the order (highest OD at "1" first): LPS E-01, LPS E-02, PS E-02, PS E-01.)

Figure 8: Indirect ELISA of LPS and of PS from *Salmonella* Choleraesuis. The antigens were coated in a 2-fold titration row starting from 0.6 µg/mL. A *Salmonella* Infantis positive

swine serum diluted 1/600 was used as antibody. (Samples appear in the order (highest OD at "1" first): LPS E-06, LPS E-08, PS E-08, PS E-06.)

Figure 9: Silver stained polyacrylamide gel of purified PS from *Salmonella* Choleraesuis.

5 1-5: Various batches of PS. 1.5 µg is applied.

Figure 10: UV spectroscopic profile of AQ-PS from *Salmonella* Typhimurium.

Figure 11: UV spectroscopic profile of AQ-PS from *Salmonella* Choleraesuis.

10

Figure 12: Silver stained polyacrylamide gel of AQ-PS from *Salmonella* Choleraesuis.

1-2: Two different batches of AQ-PS. 1.5 µg is applied.

Figure 13: Competitive ELISA of *Salmonella* Typhimurium PS 2-fold titrated on *Salmonella*

15 Typhimurium LPS-coated plate, compared to the competition with intact *Salmonella* Typhimurium LPS. Both competitive antigens were titrated from 5 mg/mL.

Plates were coated with *Salmonella* Typhimurium LPS at 0.05 µg/mL. A *Salmonella* Typhimurium positive swine serum was used as antibody at 1/400. (Upper curve: LPS, lower curve: PS.)

20

Figure 14: Competitive ELISA of *Salmonella* Choleraesuis PS 2-fold titrated on *Salmonella*

Choleraesuis LPS-coated plate, compared to the competition with intact *Salmonella* Choleraesuis LPS. Both competitive antigens were titrated from 5 mg/mL.

Plates were coated with *Salmonella* Choleraesuis LPS at 0.5 µg/mL. A *Salmonella*

25 Choleraesuis positive swine serum was used as antibody at 1/600. (Smoothest curve (and highest OD at 1 and 2): PS.)

Figure 15: Effect of AQ-PS (*Salmonella* Typhimurium) concentration on photocoupling efficiency.

30

Figure 16: Effect of AQ-PS (*Salmonella* Choleraesuis) concentration on photocoupling efficiency.

Figure 17: Effect of inorganic salts and pH on photocoupling efficiency of AQ-PS

35 (*Salmonella* Typhimurium).



Figure 18: Effect of inorganic salts and pH on photocoupling efficiency of AQ-PS (*Salmonella Choleraesuis*).

5 Figure 19: Effect of irradiation time of AQ-PS (*Salmonella Typhimurium*) and of AQ-PS (*Salmonella Choleraesuis*) on photocoupling efficiency.

Figure 20: Serological test (indirect ELISA) of pig sera on photocoupled AQ-PS (*Salmonella Typhimurium*). Sera 1 to 5: sera without any history of *Salmonella* (negative).

10 Sera 6 to 15: sera from experimentally infected pigs infected with *Salmonella Typhimurium* (positive).

Figure 21: ELISA on photocoupled mixture of AQ-PS from *Salmonella Typhimurium* and *Salmonella Choleraesuis*. Samples 1-10: Negative control sera. Samples 11-20: Positive

15 sera from experimentally *Salmonella Typhimurium* infected pigs.

Figure 22: Storage stability of photocoupled mixed AQ-PS (*Salmonella Typhimurium* and *Salmonella Choleraesuis*). Sera: White: sera without any history of *Salmonella* (negative); Red, Pink, Brown, Green: sera from experimentally infected pigs infected with *Salmonella Typhimurium* (positive); Yellow, Blue: sera from experimentally infected pigs infected with *Salmonella Infantis* (positive).

Figure 23: Indirect ELISA of PS and biotin-PS conjugates (*Salmonella Typhimurium*) on streptavidin coated plates. (Left columns: Biotin-PS; Right columns: PS.)

25

Figure 24: Two-fold titration of the APP5b PS-AQ conjugate, starting from 500 ng PS-AQ per well. The plate was incubated with an APP5b positive swine serum and a negative serum (SPF serum) diluted 1:400 in PBST.

### 30 BRIEF DESCRIPTION OF THE INVENTION

The present invention provides novel methods for immobilising a polysaccharide to a solid surface.

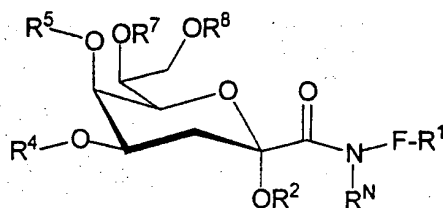
Thus, the present invention provides a method for immobilising a polysaccharide (PS) to a solid surface, said polysaccharide having a keto-carboxy group ( $-\text{C}(=\text{O})-\text{COOH}$ ) or a ketal or hemiketal group corresponding thereto), the method comprising the steps of:

- 5 a) forming a covalent bond between the carboxy group of the polysaccharide and a reporter molecule (RM), thereby forming a polysaccharide-reporter molecule conjugate (PS-RM), said reporter molecule comprising a recognition/substrate site; and
- b) immobilising the polysaccharide-reporter molecule conjugate (PS-RM) by forming a  
10 specific bond between the recognition/substrate site of said reporter molecule and a reception/reagent site of the solid surface.

The present invention also provides a solid surface thus obtainable and the use of such solid surfaces for diagnostic purposes.

15

Furthermore, the present invention provides a compound of the general formula I



(or, in the case where R<sup>2</sup> is hydrogen, optionally the keto analogue thereof)  
wherein

- 20 R<sup>1</sup> is selected from hydrogen and a reporter molecule L-R, where L is an optional linker part of the reporter molecule, and R is a report part of the reporter molecule;  
R<sup>N</sup> is selected from hydrogen and C<sub>1-4</sub>-alkyl;  
F is selected from a single bond, phenylene, carbonyl (C(=O)), carbonylimino (C(=O)-NH-), thiocarbonyl ((C(=S)) and imino (-NH-);
- 25 R<sup>2</sup>, R<sup>7</sup>, R<sup>8</sup> are each independently selected from hydrogen and hydroxy protecting groups;  
R<sup>4</sup> is selected from hydrogen, a mono- or disaccharide residue and a hydroxy protecting group; and  
R<sup>5</sup> is selected from hydrogen, an "optionally functional group protected polysaccharide residue" and a hydroxy protecting group,

30

and a method for preparation thereof.

It is, thus, a special object of the present invention to provide a method for the regioselective coupling of LPS derived bacterial polysaccharides to solid surfaces, said method being generic for all types of such LPS derived bacterial polysaccharides, said method further having no influence on the antigenic structure of the polysaccharide and finally, not introducing components adding to the non-specific background or undesired cross-reactivity when the immobilised polysaccharide is used in an assay.

## 10 DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the present invention, i.a., relates to a method for immobilising a polysaccharide (PS) to a solid surface.

15 In the present context, the term "polysaccharide" is intended to mean an entity comprising two or more glycoside linked monosaccharide units. Preferably the "polysaccharide" comprises at least 5, e.g. 5-1000, linked monosaccharide units, such as at least 10, e.g. 10-1000, linked monosaccharide units, in particular at least 25, e.g. 25-500, linked monosaccharide units. The polysaccharide may be linked so as to form a linear or  
20 branched polysaccharide.

The individual monosaccharides are typically naturally-occurring monosaccharides known to the person skilled in the art as constituents in polysaccharides of natural origin. Examples of such monosaccharides are ribose, deoxyribose, arabinose, xylose, apiose, fucose, rhamnose, fructose, glucose, mannose, galactose, glucosamine, muramic acid, galactosamine, glucuronic acid, iduronic acid, mannuronic acid, guluronic acid, galactoronic acid, *glycero-manno*-heptose, 3-deoxy-*manno*-octulosonic acid, neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid), abequose, N-acetyl-galactosamine, and N-acetyl-galactofuranose. Particularly interesting examples are D-  
25 ribose, D-deoxyribose, D-arabinose, D-xylose, D-apiose, L-fucose, L-rhamnose, D-fructose, D-glucose, D-mannose, D-galactose, L-galactose, D-glucosamine, muramic acid, D-galactosamine, D-glucuronic acid, L-iduronic acid, D-mannuronic acid, L-guluronic acid, D-galactoronic acid, L-*glycero*-D-*manno*-heptose, 3-deoxy-D-*manno*-octulosonic acid (KDO), neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid),  
30 abequose, N-acetyl-D-galactosamine, and N-acetyl-D-galactofuranose. Most interesting

are D-glucosamine, KDO, L-glycero-D-manno-heptose, D-glucose, D-galactose, N-acetyl-D-glucosamine, L-rhamnose, and D-galactofuranose.

The polysaccharide may be essentially unsubstituted but due to the typical biological origin of the polysaccharide, it is typically non-stoichiometrically substituted with acetyl, phosphate, pyrophosphate, phosphoethanolamine, ethanolamine pyrophosphate, O-methyl, sulphate, or other groups.

The molecular weight of the polysaccharide (including any substituents) is preferably at least 1,000, e.g. in the range of 1,000-200,000, in particular at least 2,000, e.g. in the range of 2,000-200,000, especially at least 5,000.

Naturally-occurring bacterial lipopolysaccharides are normally present as a mixture of molecules differing in the number of repeating units and, consequently in molecular weight as has been repeatedly shown with lipopolysaccharides in some bacterial strains consisting of molecules with none to 40 repeating units, covering molecular weights from below 10 to above 100 kD (Goldman and Leive, 1980, Eur. J. Biochem. 107, 145-153). (A repeating unit is the mono-saccharide or more commonly the oligosaccharide being repeated in the O-polysaccharide (see e.g. Figure 1). Examples of such interesting oligosaccharide repeating units include the tetrasaccharide repeating unit of *Salmonella* Typhimurium and the acetylated pentasaccharide unit of *Escherichia coli* K12 (Raetz 1996).) Commonly, molecular weights of LPS derived polysaccharides may lay in the range of 2 to 50kD, corresponding to 0 to 40 repeating units, often with a bimodal distribution with an overrepresentation of molecules with zero and one repeating unit and of molecules with 25-35 repeating units, respectively (Raetz 1996). It is often advantageous to retain the molecular weight distribution of the natural LPS in order to reflect the exact antigenicity of the intact O-antigens.

Thus, it should be understood that the above-stated ranges for numbers of monosaccharide units and molecular weights relate to the average number of monosaccharides found in the naturally occurring LPSs.

The polysaccharide which is to be immobilised comprises a keto-carboxy group (-C(=O)-COOH) or a ketal or hemiketal group corresponding thereto) which is a crucial anchor point for establishment of the link to the solid surface. In many instances (and preferably), this keto-carboxy group is provided by a KDO (2-keto-3-deoxy-D-manno-octonic acid)

monosaccharide unit included in the polysaccharide. One or more KDOs are present in the inner core oligosaccharide of all known Gram-negative lipopolysaccharides as one or more (typically 3; see Figure 1) linked monosaccharides. One of these KDOs comprises the glycosidic bond to the hexosamine dimer of the lipid A and one, being the same KDO  
5 monosaccharide or another KDO monosaccharide comprises the glycosidic bond to the reducing end of the rest of the polysaccharide part of the lipopolysaccharide.

Figure 1 illustrates a typical Gram-negative bacterial lipopolysaccharide from *E. coli* K-12 (from Raetz 1996).

10

The polysaccharides for which the present invention is especially applicable are polysaccharides which are substantially identical to the carbohydrate part of a Gram-negative bacterial lipopolysaccharide (LPS). By the "carbohydrate part of a Gram-negative bacterial lipopolysaccharide" is meant the entire polysaccharide chain, or "tree",  
15 of the lipopolysaccharide except the monosaccharides derivatised with fatty acids in ester- and/or amide bonds comprising the lipid A part. Furthermore, the carbohydrate part does not necessarily comprise all KDO units of the entire polysaccharide chain (see below).

By "substantially identical" is preferably also meant that the polysaccharide has  
20 substantially the same biological binding activity as the carbohydrate part of the native lipopolysaccharide. This means that the outer core part and the O-antigen (the serotype specific part) of the polysaccharide is preferably substantially identical to that of the native bacterial lipopolysaccharide, whereas some inner core monosaccharide (e.g. one or two, but not all, KDO units) may have been chemically cleaved off when the carbohydrate part  
25 is chemically cleaved from the lipid A part (see Figure 2 for the possible points of attack of a lipopolysaccharide (LPS) leading to a polysaccharide (PS)). It is important to note that the feature of the polysaccharide is that the serotype specificity is substantially preserved, i.e. that the O-antigen portion of the native lipopolysaccharide (LPS) is substantially unaffected by the cleavage.

30

The inner core part of a lipopolysaccharide polysaccharide is defined as the well-conserved KDO and heptose-containing reducing oligosaccharide of Gram-negative bacterial lipopolysaccharide, said oligosaccharide containing the linkage between KDO and lipid A.

35

Lipid A is the well-conserved part of bacterial lipopolysaccharides comprising fatty acids bound to a substituted hexosamine dimer, which, in turn, is the unit bound to the reducing KDO of the inner core in unmodified lipopolysaccharide.

- 5 Preferably, the polysaccharide is obtained (or at least obtainable) by the selective hydrolysis of the ketal bond between the inner core part and the lipid A part of a Gram-negative bacterial lipopolysaccharide. Preferably substituents on the polysaccharide like acetyl, phosphate, pyrophosphate, phosphoethanolamine, ethanolamine pyrophosphate, O-methyl, sulphate, or other groups are not cleaved during the selective hydrolysis.

10

"Selective hydrolysis" means a chemically or enzymatically mediated hydrolysis preferentially cleaving a selected glycoside bond, i.e. in the present context, the bond between the lipid A part and the KDO part.

- 15 Although the present invention is applicable (and advantageous) for polysaccharides (PS - i.e. polysaccharides without a lipid part) derived from Gram-negative bacterial lipopolysaccharides (LPS), it should be understood that it is believed realistic and possible to use the complete lipopolysaccharide (LPS) of Gram-negative bacteria in the method according to the invention with a fair result. This is believed to be possible due to the fact
- 20 that the lipopolysaccharide as such comprises the necessary keto-carboxy group. This constitutes a separate embodiment of the present invention. Thus, the present invention also provides a method, wherein the polysaccharide further comprises the lipid part of a Gram-negative bacterial lipopolysaccharide, i.e. the "polysaccharide" thereby constituting a bacterial lipopolysaccharide.

25

The bacteria from which the polysaccharides (and thereby also the lipopolysaccharides) are derived are preferably Gram-negative bacteria that are human or veterinary pathogens such as enterobacteria, respiratory bacteria, urogenital bacteria, and neuropathogenic bacteria.

30

- Examples of bacteria which give rise to especially applicable polysaccharides within the present invention are selected from human Gram-negative bacteria comprising *Haemophilus sp*, *Escherichia coli ssp*, *Salmonella sp*, *Klebsiella sp*, *Bordetella sp*, *Pseudomonas sp*, *Chlamydia sp*, *Neisseria sp*, *Vibrio cholerae*, *Shigella sp*, *Proteus sp*,
- 35 *Brucella sp*, *Streptobacillus sp*, *Yersinia sp*, *Legionella pneumophila*, and *Serratia*

- marcescens*, especially *Haemophilus influenzae*, *Salmonella enterica* ssp., *Klebsiella pneumoniae*, *Bordetella pertussis*, *Pseudomonas aeruginosa*, *Chlamydia psittaci*, *Neisseria meningitidis*, *Neisseria gonorrhea*, *Vibrio cholerae*, *Shigella flexneri*, and *Shigella dysenteriae*, and veterinary or zoonotic bacteria including enterobacteria selected from
- 5 *Escherichia coli*, *Salmonella* Typhimurium, *Salmonella* Choleraesuis, *Salmonella enterica*, and all serotypes hereof, especially *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Choleraesuis, *Salmonella* Manhattan, *Salmonella* Dublin, *Salmonella* Infantis, *Escherichia coli* spp. including O157, oedema-disease causing *Escherichia coli*, *Yersinia enterocolitica*, and *Campylobacter jejuni*, as well as respiratory bacteria selected from the
- 10 HAP group of bacteria especially *Actinobacillus* sp, in particular the HAP group bacteria *Actinobacillus pleuropneumoniae*, *Haemophilus somnus*, *Pasteurella haemolytica*, *Pasteurella multocida*, *Haemophilus parasuis* and *Mannheimia* sp.

#### Isolation of LPS

15

LPSs are generally isolated from Gram-negative bacteria by aqueous phenol extraction followed by various purification steps (Raetz 1996). These methods can in general be applied to all types of bacterial LPS. Also, LPSs are commercially available.

#### 20 Characterisation of LPS

- Isolated LPS can be characterised by a number of different methods as described in the Experimental section. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) can be used to analyse the molecular weight distribution of the LPS. Both
- 25 smooth and rough LPSs including commercially available materials can be analysed by this method. Also mass spectrometry, NMR, indirect ELISA (see the Experimental section), UV-spectrometry (see the Experimental section), immuno-blotting and other methods can be used.

#### 30 Preparation of PS

- It is well-known that the intact O-antigen can be isolated from the LPS by cleavage of the O-polysaccharide from the lipid A part. This is routinely done by acidic hydrolysis (e.g. 0.1 M acetic acid at 90°C for 1 hour) of the acid-labile glycoside bond between the
- 35 reducing ends of the KDO in the O-polysaccharide, and the GlcNAc in the lipid A-part of

LPS, followed by aqueous chloroform/methanol extraction to afford the polysaccharide (PS) (Raetz 1996).

The isolated PS contains the (substantially) intact O-antigen of the LPS. Elimination of the lipid A from the PS facilitates the accessibility of antibodies to the O-antigen (Munford and Hall, 1979, Inf. Imm. 26, 42-48) as well as decrease the endotoxicity of the O-antigen about 1000 times relatively to the corresponding LPS (van de Wiel et al., 1987, Vaccine 5, 33-38). In addition, PSs are pure hydrophilic molecules, they are of low toxicity, highly soluble in aqueous solutions and show no tendency to form micelles. PSs are therefore easier to handle than LPSs.

Other methods that may be used to obtain delipidated LPS polysaccharides include alkaline hydrolysis by hydrazine or NaOH, releasing esterified fatty acids in addition to phosphate and other esters, but not amide-bound fatty acids (Gupta et al. 1995) and phage-mediated degradation of the O-polysaccharide of intact LPS or PS into smaller oligosaccharide units, not retaining the terminal KDO monosaccharide (Svenson et al. 1979, J. Virol. 32, 583-592). In addition to the phase-extraction method employed in the examples it is well-known that PS can be worked up from acid hydrolysis by gel permeation chromatography (Lambden and Heckels, 1982).

### Characterisation of PS

Isolated PS can be characterised by a number of different methods as described in the Experimental section. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) can be used to analyse for remaining LPS. Also mass spectrometry, NMR, indirect and competitive ELISA (see the Experimental section), UV-spectrometry (see the Experimental section), immuno-blotting and other methods can be used.

### Preparation of polysaccharide reporter group conjugates

To obtain the best performance of an immobilised polysaccharide antigen in a diagnostic assay or other applications, it is necessary to immobilise the antigen in a well defined orientation on the solid surface. Within the present invention, this requires the regiospecific formation of a chemically stable bond between a reporter molecule and the polysaccharide antigen.



As mentioned above, the method of the present invention includes the step of forming a covalent bond between the carboxy group of the polysaccharide (PS) and a reporter molecule (RM), thereby forming a polysaccharide-reporter molecule conjugate (PS-RM),  
5 wherein said reporter molecule comprises a recognition/substrate site.

The term "reporter molecule" is intended to mean a chemical entity which is used to establish the specific link between the polysaccharide and the solid surface. The reporter molecule comprises at least a reporter part which includes the recognition/substrate site,  
10 and optionally a linker part.

The polysaccharide-reporter molecule conjugate (PS-RM) preferably has the general formula  $PS'-C(=O)-N(R^N)-F-L-R$ , where  $PS'-C(=O)$  is the polysaccharide,  $N(R^N)-F$  is the group directly involved in the covalent link between the polysaccharide and the reporter  
15 molecule,  $R^N$  designates hydrogen or  $C_{1-4}$ -alkyl, L is the linker part of the reporter molecule, and R is the reporter part of the reporter molecule. In particular, -N-F- designates amino (-N-), anilino (-N-Ph), hydrazido (-N-C(=O)-), semicarbazido (-N-C(=O)-NH-), thiosemicarbazido (-N-C(=S)-NH-), or hydrazino (-N-NH-). Preferably -N-F- is amino.

20 The term " $C_{1-4}$ -alkyl" is intended to cover methyl, ethyl, propyl (1-propyl and 2-propyl), cyclopropyl, butyl (1-butyl, 2-butyl, 2-methyl-prop-1-yl and 2-methyl-prop-2-yl (tert-butyl)).

Various types of reporter parts are generally described below under "Immobilisation of  
25 polysaccharide reporter molecule conjugates".

Examples of reporter parts are photochemically reactive groups such as substituted coumarins, benzofurans, indols, angelicins, psoralens, carbene and nitrene precursors, ketones, and quinones, e.g. anthraquinones (AQ), phenanthraquinones and  
30 benzoquinonones; thermochemically reactive groups such as carboxylic acids, primary amines, secondary amines, acid hydrazides, semicarbazides, thiosemicarbazides, thiols, aliphatic hydrazines, aromatic hydrazines, epoxides and maleimides; and one part of an affinity pair (preferably the part having the lower molecular weight, e.g. a molecular weight of up to 7,000) such as one part of biotin/avidin, biotin/streptavidin, biotin/NeutrAvidin™,  
35 glutathione/glutathione-S-transferase, iminodiacetic acid metal complex/hexa-histidine

tagged peptides and proteins, nitrilotriacetic acid metal complex/hexa-histidine tagged peptides and proteins, LNA/LNA, LNA/DNA, LNA/RNA, DNA/DNA, DNA/RNA, RNA/RNA, PNA/RNA, PNA/DNA and mono- or polyclonal antibodies raised against a specific hapten/hapten. Preferably, the reporter part comprises a biotin (a part of an affinity pair) or  
5 an anthraquinone.

The reporter molecule may also include a suitable linker part (L) in addition to a reporter part. Such linker may be useful in providing sufficient flexibility/mobility of the immobilised polysaccharide.

10

The optional linker part between the reporter molecule and the polysaccharide can be used for different purposes. The linker part may be used to space the reporter group from the polysaccharide thus, enhancing the subsequent immobilisation step. At the same time the spacer also enhances the presentation of the polysaccharide epitopes improving an  
15 diagnostic assay or other applications based on the immobilised polysaccharide. Appropriate linkers may also provide charged, uncharged, hydrophilic or hydrophobic moieties as desired influencing e.g. the solubility of the reporter group spacer molecule during conjugation to the polysaccharide as well as the properties of the final application of the immobilised polysaccharide. Thus, appropriate conjugate design and optimised  
20 conjugation protocols are key elements in the ability to produce optimal polysaccharide reporter group conjugates for the subsequent immobilisation to solid surfaces.

Examples of linker parts are biradicals selected from  $C_{1-20}$ -alkylene optionally comprising aromatic or mono-/polyunsaturated hydrocarbons or cyclic hydrocarbons, oligo-  
25 oxyethylenes, oligo-amides such as oligo-glycine, oligo-alanine, oligo-lysine and oligopeptides in general, oligo-phosphodiester, oligo-phosphoamidates, oligo-phosphodiamides, oligo-sulfonesters, and oligo-sulfonamides. Moreover, the linker may also consist of combined units of the aforementioned.

30 It is preferred that the linker part, if present, introduces 1-30 atoms, preferably 3-20 atoms, between the F and R in the above formula  $PS'-C(=O)-N(R^N)-F-L-R$  (and in the above formula I)

Preferably, the reporter molecule has a molecular weight of at the most 10,000, such as at  
35 the most 5,000, preferably at the most 2,500.

The term "recognition/substrate site" of the reporter part of a reporter molecule is intended to mean the site being, or including, a (biologically) recognition site (i.e. one part of an affinity pair) or a chemically reactive part or a photochemically reactive part which is intended to form a specific bond with a reception/reagent site of the solid surface.

As mentioned above, it has been found that the carboxylic acid in a keto-carboxy containing monosaccharide unit in a polysaccharide is a especially good handle for regiospecific as well as chemoselective bond formation between the polysaccharide and a reporter molecule containing a suitable functional group.

Several nucleophiles including primary and secondary amines, aliphatic hydrazines, aromatic hydrazines, semicarbazides, thiosemicarbazides and acid hydrazides are able to form a stable covalent bond with a carboxylic acid moiety with the aid of a coupling reagent. Thus, reporter groups containing such nucleophilic groups will be able to form stable covalent bonds with a carboxylic acid. It should be understood that some "reporter parts" may in themselves comprise such a nucleophile. Alternatively such a nucleophile may be introduced directly or via the optional linker part (L). Preparation of such nucleophile containing reporter molecules is well-known to the person skilled in the art (see e.g. Greg T. Hermanson et. al., Immobilized Affinity Ligand Techniques, Academic Press Inc., 1992).

The coupling reagent acts as an activator of the carboxylic acid and a subsequent attack by the nucleophile leads to the formation of a stable covalent bond. Examples of coupling reagent are carbodiimides such as diisopropyl carbodiimide and water soluble carbodiimide (WSC, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide), phosphonium salts such as benzotriazolyloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and (benzotriazolyl)-N-oxy-pyrrolidinium phosphonium hexafluorophosphate (PyBOP), uronium salts such as O-benzotriazolyl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), O-(3,4-dihydro-4-oxo-1,2,3-benzotriazinyl) N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU), and N, N'-Tetramethyl flouroformimidinium hexafluorophosphate (TFFH). Many other coupling reagents may be used and are known to the person skilled in the art (M. Bodanszky, Principles of Peptide Synthesis, 2nd Edition, Springer-Verlag, 1993). The addition of helper nucleophiles such as N-hydroxy succinimide and 1-hydroxy benzotriazole may enhance the coupling kinetics as well as

suppressing side reactions such as O->N acyl rearrangements when using carbodiimides as coupling reagents (M. Bodanszky, Principles of Peptide Synthesis, 2nd Edition, Springer-Verlag, 1993).

- 5 The coupling reactions can be performed in aqueous solution or in a mixture of water and an water miscible organic solvent such as N,N-dimethyl formamide, dimethyl sulfoxide, dioxane, methanol, ethanol and acetone or in the pure organic solvent. The solvent or solvent mixture is selected to allow the best possible solubility of the reactants.
- 10 When using water or a water organic solvent mixture the pH during the coupling reaction can be controlled by using a buffer solution. The pH is selected to allow the best conditions for the coupling reaction while at the same time preserving the integrity of the polysaccharide and reporter molecule. Preferentially the pH is kept between 5 and 9 in order to preserve acid or base labile epitopes on the O-chains of the polysaccharide. An
- 15 example of such a labile group is O-acetyl functionalities which are very commonly found in polysaccharide O-antigens. The optimum pH for the coupling reaction also depends on the nature of the nucleophile. Amines are basic compounds that are protonised at low pH which renders them non-nucleophilic and thus, coupling reactions using amines are best performed at a pH above 7. Other nucleophiles such as aromatic hydrazines,
- 20 semicarbazides, thiosemicarbazides and acid hydrazides are much less basic allowing the coupling reaction to be performed at neutral pH or even lower. Another factor which determines the optimum pH for the coupling reaction is the competing hydrolysis of the activated carboxylic acid moiety by the attach of hydroxide ions. This side reaction can be minimised by the use of a pH below 8 during coupling. In organic solvents the pH during
- 25 the coupling reaction can be achieved by the use of suitable organic or inorganic acids and bases that are selected according to their solubility in the solvent as well as according to their relative acid or base strength. As described above, the precise conditions are chosen as promote the covalent coupling while at the same time preserving the integrity of the polysaccharide and reporter molecule.
- 30 The coupling reactions are typically performed at a temperature in the range of -20-100°C, often 0-20°C such as around 5°C.

In addition to the above mentioned parameters reaction time as well as the amount of

35 polysaccharide, coupling reagent and reporter group are critical for obtaining the ideal

polysaccharide reporter group conjugate. The inventors have developed coupling protocols which yield reproducible conjugates in excellent yields and quality preserving the immunogenic epitopes of the polysaccharide as well as the integrity of the reporter group (see the Experimental section). Such optimisation is often desirable for commercial applications as will be apparent for the person skilled in the art.

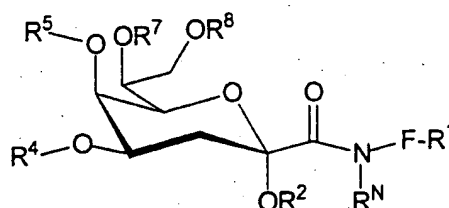
In an interesting and commercially very interesting embodiment of the present invention, the polysaccharide is a polysaccharide derived from Salmonella LPS, and the reporter molecule which is coupled to the polysaccharide via an amide bond by means of a carbodiimide coupling reagent comprises a biotin or anthraquinone group.

Another an interesting and commercially very interesting embodiment of the present invention, the polysaccharide is a polysaccharide derived from Actinobacillus LPS, and the reporter molecule which is coupled to the polysaccharide via an amide bond by means of a carbodiimide coupling reagent comprises a biotin or anthraquinone group.

These embodiments are thoroughly discussed in the Experimental section.

It is believed that the intermediates produced according the first step of the method according to the present invention are novel as such.

Thus, the present invention also provides a compound of the general formula I



(or, in the case where  $R^2$  is hydrogen, optionally the keto analogue thereof)

wherein

$R^1$  is selected from hydrogen and a reporter molecule L-R, where L is an optional linker part of the reporter molecule, and R is a report part of the reporter molecule;

$R^N$  is selected from hydrogen and  $C_{1-4}$ -alkyl;

F is selected from a single bond, phenylene, carbonyl ( $C(=O)$ ), carbonylimino ( $C(=O)-NH-$ ), thiocarbonyl ( $C(=S)$ ) and imino ( $-NH-$ );

$R^2$ ,  $R^7$ ,  $R^8$  are each independently selected from hydrogen and hydroxy protecting groups;  
 $R^4$  is selected from hydrogen, a mono- or disaccharide residue and a hydroxy protecting group; and  
 $R^5$  is selected from hydrogen, an "optionally functional group protected polysaccharide residue" and a hydroxy protecting group.

The term "mono- or disaccharide residue" is intended to mean a residue comprising one or two glycosidically linked monosaccharide units. Preferred examples are KDO-2-yl, 4-phosphoethanolamine-KDO-2-yl, L-rhamnosyl-(1->4)-KDO-2-yl, and KDO-(2->4)-KDO-2-yl.

The term "polysaccharide residue" is intended to mean the part of a polysaccharide (as defined above) which together with the KDO monosaccharide unit of formula I and any  $R^4$  saccharide substituents forms the polysaccharide (PS). Thus, the polysaccharide residue preferably comprises from 2-997 linked monosaccharide units, such as at least 7, e.g. 7-997, linked monosaccharide units, in particular at least 22, e.g. 22-497, linked monosaccharide units. As defined above for "polysaccharide", the polysaccharide residue may be non-stoichiometrically substituted.

The term "optionally functional group protected" is intended to mean that the oligosaccharide fragment which carries functional groups which are reactive under the conditions prevailing in coupling steps, are optionally functional group protected as known in the art. This means that groups such as hydroxy, amino, carboxy, sulphonyl, and mercapto groups are optionally functional group protected. Protection (and deprotection) is performed by methods known to the person skilled in the art (see, e.g., Greene, T. W. and Wuts, P. G. M., "Protective Groups in Organic Synthesis", 2<sup>nd</sup> ed., John Wiley, N.Y. (1991), and M.J. Gait, Oligonucleotide Synthesis, IRL Press, 1984).

Illustrative examples of "hydroxy protection groups" are optionally substituted trityl, such as 4,4'-dimethoxytrityl (DMT), 4-monomethoxytrityl (MMT), and trityl, optionally substituted 9-(9-phenyl)xanthenyl (pixyl), optionally substituted ethoxycarbonyloxy, *p*-phenylazophenylloxycarbonyloxy, tetraahydropyranyl (thp), 9-fluorenylmethoxycarbonyl (Fmoc), methoxytetrahydropyranyl (mthp), silyloxy such as trimethylsilyl (TMS), triisopropylsilyl (TIPS), *tert*-butyldimethylsilyl (TBDMS), triethylsilyl, and phenyldimethylsilyl, benzyloxycarbonyl or substituted benzyloxycarbonyl ethers such as 2-bromo

benzyloxycarbonyl, *tert*-butylethers, alkyl ethers such as methyl ether, acetals (including two hydroxy groups), acyloxy such as acetyl or halogen substituted acetyls, e.g. chloroacetyl or fluoroacetyl, isobutyryl, pivaloyl, benzoyl and substituted benzoyls, methoxymethyl (MOM), benzyl ethers or substituted benzyl ethers such as 2,6-dichlorobenzyl (2,6-Cl<sub>2</sub>Bzl). Alternatively, a hydroxy group may be protected by attachment to a solid support optionally through a linker.

Illustrative examples of amino protection groups are Fmoc (fluorenylmethoxycarbonyl), BOC (*tert*-butoxycarbonyl), trifluoroacetyl, allyloxycarbonyl (alloc, AOC), benzyl-oxycarbonyl (Z, Cbz), substituted benzyloxycarbonyls such as 2-chloro benzyloxycarbonyl ((2-ClZ), monomethoxytrityl (MMT), dimethoxytrityl (DMT), phthaloyl, and 9-(9-phenyl)xanthenyl (pixyl).

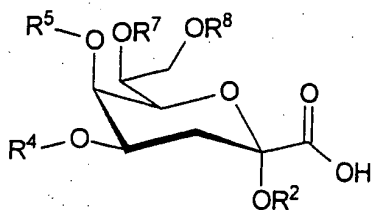
Illustrative examples of carboxy protection groups are allyl esters, methyl esters, ethyl esters, 2-cyanoethylesters, trimethylsilylethylesters, benzyl esters (Obzl), 2-adamantyl esters (O-2-Ada), cyclohexyl esters (OcHex), 1,3-oxazolines, oxazoles, 1,3-oxazolidines, amides and hydrazides.

Illustrative examples of mercapto protecting groups are trityl (Trt), acetamidomethyl (acm), trimethylacetamidomethyl (Tacm), 2,4,6-trimethoxybenzyl (Tmob), *tert*-butylsulfenyl (StBu), 9-fluorenylmethyl (Fm), 3-nitro-2-pyridinesulfenyl (Npys), and 4-methylbenzyl (Meb).

In an interesting embodiment, R<sup>1</sup> is L-R. Furthermore, -N-F- in the formula I preferably designates amino (-N-), anilino (-N-Ph), hydrazido (-N-C(=O)-), semicarbazido (-N-C(=O)-NH-), thiosemicarbazido (-N-C(=S)-NH-) or hydrazino (-N-NH-), in particular amino.

The group L preferably designates a biradical (linker part) as defined above and, furthermore, the reporter molecule (L-R) is preferably also as defined above. Variants where the reporter molecule comprises a biotin or an anthraquinone (in particular an anthraquinone) as a part of the reporter part are especially interesting.

Furthermore, the present invention provides a method for the preparation of a compound of the general formula I as defined above, comprising reacting a compound of the general formula III



III

5 wherein  $R^2$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^7$  and  $R^8$  are as defined for formula I, in fully or partly functional group protected form,

with a nitrogen compound of the general formula IV



wherein  $R^1$ , F and  $R^N$  are as defined for formula I;

and optionally fully or partly deprotecting the product thereby obtained in order to obtain a  
15 compound of the general formula I.

Preferably the compound of the general formula III is used in its activated ester form. In particular, the reaction between the carboxylic acid compound of the general formula III and the nitrogen compound IV is facilitated by using a coupling reagent such as one of  
20 those mentioned further above.

Thus, the present invention also provides a compound of the formula I for the preparation of an assay device for the detection of antibodies against Gram-negative bacteria and/or O-antigens of Gram-negative bacteria.

25

The present invention further provides a compound of the formula I for the preparation of a solid surface carrying an immobilised polysaccharide.



### Analysis of polysaccharide reporter group conjugates

It is often valuable to be able to assess the quality of the reporter group polysaccharide conjugates prior to immobilisation to solid surfaces. Appropriate analytical protocols must  
5 be available to ensure that immunogenic epitopes on the polysaccharide are intact after conjugation to the reporter molecule and at the same time to ensure that the conjugates are produced in a reproducible manner.

In connection with the present invention, a UV spectroscopic method that can be used to  
10 assess the amount of reporter groups attached to the polysaccharide as well as to assess the amount of DNA and protein present in the polysaccharide reporter group preparations has been developed. Thus, as described in the Experimental section, measurement of the absorption at 260 and 280 nm can be used to assess the amount of DNA and protein, while the amount of reporter groups attached to the polysaccharide can be assessed at a  
15 wavelength specific to the reporter group. Competitive ELISA and indirect ELISA as described in the Experimental section is a powerful tool to quantify the presence of intact immunogenic epitopes in different polysaccharide reporter group conjugate preparations. Thus, the combination of the two developed analytical techniques ensures that different batches of polysaccharide reporter group preparations can be quality controlled. The final  
20 decisive analytical protocol is the quantitative measurement of the ability to immobilise of the polysaccharide reporter group conjugates. These procedures are outlined below and in the Experimental section.

### Immobilisation of polysaccharide reporter molecule conjugates

25

In a subsequent step of the method according to the present invention, the polysaccharide-report molecule conjugate is immobilised to the solid surface by forming a specific bond between the recognition/substrate site of said reporter molecule and a  
reception/reagent site of the solid surface.

30

The term "forming a specific bond" is intended to mean the establishment of a covalent bond between the reporter part of the polysaccharide/reporter molecule conjugate and the solid surface as well as establishment of a non-covalent bond involving an affinity pair.

In one aspect of this invention the reporter part is a photochemically reactive group that is capable of forming a covalent bond to a solid surface upon irradiation with light. Examples of photochemically reactive groups are substituted coumarins, benzofurans, indols, angelicins and in particular psoralens as disclosed in EP 0 319 957. A number of patent  
5 publications US-A-4 722 906, US-A-4 973 493, US 5 002 582 and PCT/US88/04491 disclose photochemically reactive groups selected from carbene and nitrene precursors and ketones that are able to form covalent bonds to solid surfaces upon irradiation with light. A particular preferred subclass of photochemically reactive groups are quinones such as those disclosed in WO 96/31557, e.g. anthraquinones (AQ), benzoquinones and  
10 phenanthraquinones. A particularly interesting photochemically reactive group is the anthraquinone group.

The photochemical formation of the covalent bond to the solid surface typically comprises of the following steps: The polysaccharide reporter molecular conjugate (PS-RM) is  
15 brought into solution in an appropriate solvent. Preferentially, the solvent is water, or a mixture of water and a water miscible organic solvent such as N,N-dimethyl formamide, dimethyl sulfoxide, dioxane, methanol, ethanol and acetone or the pure organic solvent. The solvent or solvent mixture is selected to ensure the solubility of the polysaccharide reporter molecule conjugate but at the same time allowing the best conditions for the  
20 subsequent immobilisation step. Using water or a mixture of water and a water miscible organic solvent, inorganic salts may be added to enhance the photochemical coupling step to the solid surface and to control the pH of the solution. As demonstrated in the Experimental section the exact nature of the salts as well as the concentration of the polysaccharide reporter molecule conjugate and pH of the solution are parameters which  
25 can be adjusted in order to obtain improved results. Often, the photochemical process require (or is facilitated by) a carbon-containing surface.

The solution of the polysaccharide reporter molecule conjugate is brought into contact with the solid surface while still in solution, and is exposed to light of a suitable  
30 wavelength. Preferentially, the wavelength is chosen between 200 and 700 nm but depends on the specific photochemically reactive group chosen. For anthraquinones, the wave length is typically 300-400 nm. Irradiation time varies depending on the nature of the polysaccharide reporter molecule conjugate but preferentially irradiation times should be less than 200 minutes. Alternatively, the solution of the polysaccharide reporter molecule  
35 conjugate is brought into contact with the solid surface, the solvent or solvent mixture is

evaporated and the conjugate is finally exposed to light as described above. In both instances rinsing with water or a suitable water/solvent mixture is used afterwards to remove non-covalently bound conjugates.

- 5 In a second aspect of this invention the reporter part is a thermochemically reactive group that is capable of forming a covalent bond in a chemoselective manner to a solid surface having a suitable functional group. Examples of thermochemically reactive groups are carboxylic acids, primary amines, secondary amines, acid hydrazides, semicarbazides, thiosemicarbazides, thiols, aliphatic hydrazines, aromatic hydrazines, epoxides and  
10 maleimides. Often, the thermochemical process require (or is facilitated by) a carbon-containing surface.

The polysaccharide reporter molecule conjugate (PS-RM) is brought into solution in an appropriate solvent. Preferentially the solvent is water, or a mixture of water and an water  
15 miscible organic solvent such as N,N-dimethyl formamide, dimethyl sulfoxide, dioxane, methanol, ethanol and acetone or the pure organic solvent. The solvent or solvent mixture is selected to ensure the solubility of the polysaccharide reporter molecule conjugate but at the same time allowing the best conditions for the subsequent immobilisation step. The solution of the polysaccharide reporter molecule conjugate is brought into contact with the  
20 solid surface in order to facilitate the covalent bond formation. Addition of additional coupling reagents may be necessary in order to promote the covalent bond formation. In most cases it is very important to control the pH during the covalent bond formation. In aqueous solution or in mixture of water and a water miscible solvent this can be achieved using standard buffers. In organic solvents this can be achieved by the use of suitable  
25 organic or inorganic acids and bases that are selected according to their solubility in the solvent as well as according to their relative acid or base strength. In all cases the precise conditions are chosen to promote the covalent coupling while at the same time preserving the integrity of the polysaccharide and the reporter molecule. In most cases the covalent bond formation is allowed to proceed without evaporation of the solvent, but in certain  
30 cases evaporation can be advantageous. Many varieties of such methods exist and are well known to the person skilled in the art (Greg T. Hermanson et. al., Immobilized Affinity Ligand Techniques, Academic Press Inc., 1992).

In a third aspect of this invention the reporter molecule is one part of an affinity pair. Affinity  
35 pairs are well known to the person skilled in the art (Greg T. Hermanson et. al., Immobilized

Affinity Ligand Techniques, Academic Press Inc., 1992) and illustrative examples are biotin/avidin, biotin/streptavidin, biotin/NeutrAvidin, glutathione/glutathione-S-transferase, iminodiacetic acid metal complex/hexa-histidine tagged peptides and proteins, nitrilotriacetic acid metal complex/hexa-histidine tagged peptides and proteins, DNA/DNA,  
5 DNA/RNA, RNA/RNA and mono- or polyclonal antibodies raised against specific haptens/hapten. An especially intriguing example of an affinity pair is two complementary strands of locked nucleic acids (LNA, Nielsen et al., *Chem. Commun.*, 1997, 9, 825-6.). Preferably, the part of the affinity pair used to prepare the polysaccharide reporter group conjugate is a low molecular to medium molecular weight compound preferably less than  
10 10,000 most preferably less than 7,000.

The polysaccharide reporter molecule conjugate is brought into solution in an appropriate solvent. Preferentially the solvent is water, or a mixture of water and an water miscible organic solvent such as N,N-dimethyl formamide, dimethyl sulfoxide, methanol, ethanol  
15 and acetone or the pure organic solvent. The solvent or solvent mixture is selected to ensure the solubility of the polysaccharide reporter molecule conjugate but at the same time allowing the best conditions for the subsequent immobilisation step. The solution of the polysaccharide reporter molecule conjugate is brought into contact with the solid surface that has been pre-coated with the second part of the affinity pair. In most cases it  
20 is necessary control the pH, the nature and concentration of ions in the solution and in many cases it may be necessary to add detergents in order to optimise the immobilisation step. Many varieties of such methods are well known to the person skilled in the art (Greg T. Hermanson et. al., *Immobilized Affinity Ligand Techniques*, Academic Press Inc., 1992).

25

One very important property of the methods disclosed herein is that they enable the immobilisation of mixtures of more than one polysaccharide reporter group conjugate at the same time to the same surface in a well defined manner. As described in the Experimental section, the immobilisation of polysaccharide reporter group conjugates can  
30 be optimised individually and then finally be performed in a mixed reaction to create applications based on the mixed conjugates. The individual ratios of the immobilised conjugates can be easily optimised according to the specific application.

The solid surface to which the polysaccharide is to be attached can be selected from a  
35 wide variety of solid surfaces used in the analytical and diagnostic fields. The most

interesting types of solid surfaces are those of organic polymers, glasses, silicium and silicium oxide (silica) as well as composite materials thereof.

Among the organic polymers, polystyrene, polycarbonate, polypropylene, polyethylene,  
5 cellulose, nitrocellulose, agarose, polyethyleneglycol terephthalate, polyvinylacetate, polyvinylidifluoride, polymethylpentene, polyvinylpyrrolidinone, polyacrylate, polyacrylonitrile, polymethylmethacrylate and polyvinylchloride are illustrative examples, where polystyrene and polycarbonate are especially interesting examples.

10 Among the glasses and ceramics, borosilicate glass (Pyrex glass) and soda-lime glass are especially relevant examples, e.g. in the form of specimen tubes, vials, and slides for microscopy.

The body in itself may have a form or may be designed and shaped for the particular  
15 desired use. E.g. the body may be in the form of a sheet, a film, a bead, a pellet, a disc, a plate, a ring, a rod, a net, a membrane, a filter, a tray, a microplate (a microtitre plate), a stick, or a multi-bladed stick. Especially interesting bodies to be coated according to the present invention are microplates (microtitre plates), e.g. polystyrene microplates (microtitre plates), sticks, slides, tubes and beads.

20

A further important property of the methods disclosed herein is the ability to perform the immobilisation of the polysaccharide reporter group conjugates in spatially addressable manner, which is very important in the development of bio-chips and bio-sensors and other miniaturised diagnostic systems. In these systems it is necessary that at least one  
25 component of the assay is immobilised at a well defined position. This will allow that each analytical component can be identified by the position, allowing the determination of many analytical components at the same time in one analytical sample.

It is also believed that the immobilisation of a polysaccharide may be performed according  
30 to an alternative method where a covalent bond is formed directly between the carboxy group of the polysaccharide and a chemical functionality attached to the solid surface, e.g. an amine, an aniline, a hydrazine, etc. In some instances commercially available solid surfaces may be used, e.g. the Covalink microplates from Nunc, Denmark. It is believed that this method is less advantageous than the embodiments according to the main  
35 embodiments, in particular with respect to reproducibility. It is however believed that this

alternative method might be useful for immobilisation of polysaccharides (PS) depleted the lipid part.

Thus, the present invention also provides a method for immobilising a polysaccharide (PS) to a solid surface, said polysaccharide having a keto-carboxy group ( $-C(=O)-COOH$ ) or a ketal or hemiketal group corresponding thereto), by forming a covalent bond between the carboxy group of the polysaccharide and a chemical functionality of the solid surface.

It is evident from the above that the chemical functionality of the solid surface preferably should be of the same type as the those specified above resulting in essentially the same type of bond types between the polysaccharide and the solid surface as a specified above for the bond between the polysaccharide and the reporter molecule, i.e. a bond of the type  $PS'-C(=O)-N(R^N)-F-L-SS$ , where  $PS'$ ,  $L$ ,  $R^N$  and  $F$  are as defined above and  $SS$  designates the solid surface. It should be understood that  $N(R^N)-F-L$  may be part of the solid surface or whereto the polysaccharide is coupled or, alternatively, that  $N(R^N)-F-L$  may be coupled the polysaccharide prior to the coupling to the solid surface.

In summary, the present invention also provides solid surfaces obtained (or obtainable) according to the methods described above.

#### Applications of immobilised conjugates

The solid surfaces to which LPS derived polysaccharides are immobilised have various uses within the diagnostic and analytical fields, e.g. for the detection of *Salmonella* infections in various herds of animals, e.g. swine and poultry.

In a preferred embodiment, the immobilised *Salmonella* polysaccharides comprise O-antigens 1,4,5,6,7 and 12 represented by a mixture of anthraquinone-coupled LPS-derived polysaccharides of *Salmonella* Typhimurium and *Salmonella* Choleraesuis, coupled to the solid surface as an optimised mixture of the two polysaccharides as described in Example 24. This solid surface is intended for use in an immunoassay for antibodies against *Salmonella* spp. and for *Salmonella* O-antigens of the described serotypes, the immunoassay comprising contacting the surface with a sample, preferably a liquid sample such as serum, meat juice, milk or other biologically derived fluids. Antibodies are then detected by a detection antibody that comprises an enzyme label, the

whole assay being performed as an enzyme-linked immunosorbent assay (ELISA). Other labels, e.g. fluorescent labels, labels for time-resolved fluorescent detection, radioactive labels in general, radio-labelled for proximity scintillation counting, may also be used.

- 5 The present invention provides the use of a solid surface carrying a polysaccharide immobilised as described above (or by other methods yielding the same result) in a diagnostic or antigen serotyping assay, preferably a diagnostic assay. The assay is preferably a solid phase immunoassay. In particular, the diagnostic assay is a serological assay, e.g. a serological assay for detection of antibodies against microorganisms such  
10 as Gram-negative bacteria. Especially, the diagnostic assay is a serological assay for detection of antibodies against *Salmonella* spp.

- The present invention also provides an assay device for the detection of antibodies against one or more Gram-negative bacteria, comprising a solid surface having  
15 immobilised thereto a polysaccharide, said polysaccharide corresponding to the carbohydrate part of the bacterial lipopolysaccharide (LPS) of the Gram-negative bacteria via the carboxylic acid group of a KDO monosaccharide unit of said polysaccharide. The polysaccharide is preferably immobilised to the solid surface according to the method defined herein. The solid surface which is included in the assay device typically have a  
20 shape particularly suited for the assay in question. The assay device may furthermore include a protective sheet and may be accompanied by an instructions sheet.

- Particularly interesting assay devices are those used for serotyping of bacteria and for antigen detection. Furthermore, assay devices including more than one type of PS  
25 immobilised to the surface of a device member constitute preferred embodiments.

- The present invention also provides a method for estimating the number of a specific bacteria, for serotyping of bacteria and for antigen (LPS/PS) detection by utilising the assay devices described above. Particularly interesting embodiments involve the use of  
30 AQ-PS immobilised microplates. These diagnostic methods render it possible to detect even small amounts of bacteria in biological fluids and provides the determination of a bacteria serotype. This is particularly interesting and valuable for the detection of *Salmonella* and *Actinobacillus* infections.

## EXPERIMENTAL

## General

- 5 Chloroform, dimethyl sulfoxide (DMSO) and methanol were purchased from Labscan and were of HPLC purity, acetic acid (99-100 %) from Riedel de H  en, water-soluble carbodiimide (WSC, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide) from NovaBiochem, N-hydroxy succinimide (96 %) from Aldrich, "Slide-a-lyzer" from Pierce, H-  Ala-  Ala-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl were synthesised as described earlier (WO 96/31557) (  Ala means   -alanine). Dialysis tubes (MWCO: 6-8000) were purchased from Spectra/Por, polystyrene microplates (PolySorp) from Nunc. PBS: phosphate buffered saline, pH 7.2, 0.15 M NaCl. PBS, Tween: PBS + 0.05% Tween 20. PBS, Tween, BSA: PBS-Tween + 1% BSA (bovine serum albumin)). OPD-substrate solution: 0.1M citrate-phosphate buffer, pH 5.0, 0.66 mg/mL OPD (O-phenylene diamine), 0.012% H<sub>2</sub>O<sub>2</sub>. All swine sera were from
- 10 the Danish Veterinary Laboratory.

## Example 1

**Purification of lipopolysaccharide (LPS) from *Salmonella* Typhimurium and *Salmonella* Choleraesuis**

- 20 Culture. *Salmonella* Typhimurium no. 3389-1/92 (O: 1, 4, 5, 12) and *Salmonella* Choleraesuis var. Kunzendorf no. 143 (O: 6, 7) were used for the preparation of LPS. For plate culture, Columbia agar (Oxoid, Unipath Ltd., Basingstoke, UK) supplemented with 5% bovine blood (C-blood agar) was used as the solid growth medium. For broth culture, the bacteria were grown aerobically at 37  C in flasks with shaking at 130 rpm. Strains
- 25 were stored at -80  C in LB-broth supplemented with 10% glycerol.
- For fermentation, a 7 litre MBR Labor Bioreactor (MBR Bio Reactor AG, Switzerland) with 4 litre LB medium was inoculated with a 800 mL overnight broth culture. Temperature was maintained at 37  C and pH was maintained at 7.2 throughout and aeration was set at 50% pO<sub>2</sub> automatically regulating by sparging sterile atmospheric air at constant agitation
- 30 of 500 rpm. Foam was controlled by addition of silicon emulsion. 90 minutes after inoculation, 400 mL 25% glucose was added. Five hours after inoculation, aeration was reduced to approximately 1 litre pr. minute. After approximately 18 hours of cultivation, aeration was stopped and 150 mL of formalin was added to a final concentration of approximately 3% at 20  C and 200 rpm and inactivation was continued for 20 hours under
- 35 these conditions.



Inactivation of the culture was checked on C-blood agar 6 hours after the addition of formalin, incubating the inoculated C-blood agar plates overnight. If inactivation was found to be complete the inactivated culture was released for further processing.

5

Extraction. LPS was extracted from the formalin killed cultures by hot aqueous phenol extraction, as described by Hassan et al. 1990. Briefly, the bacteria in 5 L inactivated culture broth were washed in 3x4 L PBS, followed by 4 washings in 5-10 volumes of acetone at 20°C, dried and resuspended in Milli Q water. LPS was then extracted from  
10 this suspension by hot (65°C) 45 % aqueous phenol; an equal volume of preheated 90 % aqueous phenol was added to the suspension whereafter the mixture was kept at 65-68°C for 10 min. under gentle agitation. After cooling to 4°C followed by centrifugation for 30 min at 10.000 g, the upper aqueous phase was carefully retrieved and dialysed against Milli Q water for at least 48 hrs (with 3 shifts) at 4°C and subsequently freeze-dried.

15

The average yield of purified LPS from one batch of fermentor culture was 1700 mg dry-weight with a range of 1500-2200 mg. Purity was evaluated from SDS-gels (see Example 3) and UV-analysis measurements. As seen on Figure 5 only residual amounts of proteins were seen on the gels. Extinction measurements at 280 nm showed that aqueous LPS  
20 solutions (5-10 mg/mL LPS) of LPS contained around 1.5 mg/mL protein. On silver stained SDS-PAGE (see Example 3) the typical ladder-like arrangement of bands is seen, indicating that the whole range of molecular weights has been retrieved.

## Example 2

### 25 UV-analysis of LPS

UV spectroscopy can be used to analyse the DNA and protein content of the LPS. DNA content can be measured at 260 nm and protein content at 280 nm. Briefly, the LPS is dissolved in ultra-pure water to a final concentration of 0.5 mg/mL. The UV spectrum is measured from 200 - 400 nm with ultra-pure water alone as the reference. Figure 3 shows  
30 a typical UV-profile of LPS derived from *Salmonella* Typhimurium, while Figure 4 shows a typical UV-profile of LPS derived from *Salmonella* Choleraesuis.

**Example 3****SDS-PAGE analysis of LPS**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can be used for the analysis of LPS preparations for determination of the molecular weight distribution of the preparation. By this method smooth LPS preparations typically give a "ladder" of bands representing LPS molecules of increasing molecular weight, each separated by the molecular weight of the repeating unit of the O-polysaccharide. Polyacrylamide gels (12.5%) were run according to the method of Laemmli (Laemmli, U.K., 1970, Nature 227, 680-685, Cleavage of structural proteins during the assembly of the head of bacteriophage T4). The LPS was diluted in sample buffer to a final concentration of 2-4 mg/mL LPS (1% SDS) and boiled for 5 min, before being applied to the wells. For detection of proteins the gels were stained in 0.4 % (w/w) Coomassie Brilliant Blue R250. For detection of LPS the gels were silver-stained according to the method of Tsai and Frasch (Tsai, C.-M., Frasch, C.E., 1982, Anal. Biochem. 119, 115-119, A sensitive silver stain for detecting lipopolysaccharides in Polyacrylamide gels).

Figure 5 shows a typical analysis of a *Salmonella* Typhimurium LPS preparation and Figure 6 shows a typical analysis of a *Salmonella* Choleraesuis LPS preparation. As seen in the figures, only residual amounts of protein was visualised by the Coomassie Brilliant Blue stain, while on the silver stained gels the ladder-like arrangement of bands is seen indicating that the whole range of molecular weights has been retrieved.

**Example 4****Indirect ELISA for determination of LPS and PS**

As intact LPS normally has the ability to bind passively to microplates via the hydrophobic lipid A-part of the molecule an indirect ELISA in which LPS is coated can be used to determine the amount of LPS in a certain LPS-preparation. In the same way the presence of intact LPS in a preparation of PS can be analysed by coating the PS preparation.

Indirect ELISA was performed essentially according to the protocol previously published by B. Nielsen et al.(1995), only omitting blocking of the plates. The plates were coated with a two-fold dilution series of the LPS or PS preparation typically starting at 0.05 mg/well in 0.1 M sodium carbonate, 1.0 M NaCl, pH 9.6 over night at 4°C. After washing in PBS, Tween either a standard monoclonal antibody directed against *Salmonella* Typhimurium O-antigens (MAB Hytest clone 1E6, 1 mg/mL, diluted 1/25000 in PBS, Tween, BSA) or a positive pig serum, diluted 1/400 in PBS, Tween, BSA. In Figure 7 a positive pig serum is used. 100 µL was applied pr. well and incubated for 1 h at room temperature. The plates were then washed three times in PBS, Tween and subsequently incubated with HRP-conjugated rabbit anti mouse IgG (PO260, DAKO, diluted 1:2,000) with the MAb or HRP-conjugated rabbit anti swine IgG (PO164, DAKO diluted 1:2,000) with the swine serum; dilutions were done in PBS, Tween, BSA for 1 hour at room temperature. The plates were washed as before, and 100 µL OPD substrate solution was added to each well and incubated 10-15 min. The reaction was stopped with 100 µL 0.5 M H<sub>2</sub>SO<sub>4</sub> and the optical density was read at 490 nm subtracting 650 nm for background correction.

Figure 7 shows typical example of *Salmonella* Typhimurium LPS and PS preparation analysed by a positive swine serum as described above and Figure 8 shows a corresponding analysis of a *Salmonella* Choleraesuis LPS preparation using an Infantis-positive swine serum. It is seen that the LPS-preparations bind to the plates and are specifically recognised by the antibody, allowing their quantitation when compared to a standard preparation, while PS exhibit very little binding to the plates.

25

#### Example 5

##### Competitive ELISA determination of immunogenic epitopes in LPS and PS

The presence of intact immunogenic epitopes in different LPS preparations can be determined by competitive ELISA using a fixed concentration of intact LPS for coating and incubating the antigen (LPS or PS) preparation to be investigated together with the detection antibody. Apart from the presence of competitor, the ELISA is performed exactly as the indirect ELISA described in Example 4. Typically, a 2-fold titration from 5 mg/mL of the antigen is used and the competitive effect is seen as a reduction in the OD-level compared to the OD-level obtained in the absence of competing antigen.

Figure 13 shows a typical result of the competition by a *Salmonella* Typhimurium PS preparation, using a *Salmonella* Typhimurium LPS coated microplate and a *Salmonella* Typhimurium LPS preparation as competitor control and detection by a *Salmonella* Typhimurium positive swine serum diluted 1/400 followed by HRP-conjugated rabbit-anti swine IgG (DAKO PO164, 1/2000), as detailed in Example 4. It is seen that the PS preparation at comparable concentrations is just as competitive as the intact LPS, indicating the preservation of antigenic epitopes in the PS. Figure 14 depicts the same set-up with the *Salmonella* Choleraesuis antigens, using a positive swine serum at 1/600. Again, it is seen that the antigenicity of the PS preparation is preserved compared to the intact LPS.

#### Example 6

##### Preparation of PS from LPS derived from *Salmonella* Typhimurium by delipidation

Lyophilised LPS derived from *Salmonella* Typhimurium (1.55 g) is dissolved in ultrapure water (388 mL) then acetic acid (22 mL, 0.37 mol) is added. The mixture is divided into 40 mL portions in Nunc plastic tubes. The sealed tubes are heated in an oven (90°C, 60 minutes) followed by cooling in an ice bath for 10 minutes allowing the mixtures to reach room temperature. The mixtures are pooled and the combined aqueous phases extracted with chloroform / methanol (2:1 mixture v/v, 4 x 580 mL). The aqueous phase is subsequently dialysed at 4°C against ultrapure water for 1-2 days (until the smell of phenol has disappeared) and finally lyophilised for 1-2 days giving the PS as a white solid. UV analysis (0.5 mg / mL) is performed as described in Example 2. Yield: 0.520 g (34 %), UV analysis: A(260 nm): 1.0; A(280 nm): 0.82.

#### Example 7

##### Preparation of PS from LPS derived from *Salmonella* Choleraesuis by delipidation

Lyophilised LPS derived from *Salmonella* Choleraesuis (1.13 g) is dissolved in ultrapure water (283 mL) then acetic acid (16.2 mL, 0.27 mol) is added. The mixture is divided into 40 mL portions in Nunc plastic tubes. The sealed tubes are heated in an oven (90°C, 60 minutes) followed by cooling in an ice bath for 10 minutes allowing the mixtures to reach room temperature. The mixtures are pooled and the combined aqueous phases extracted with chloroform / methanol (2:1 mixture v/v, 4 x 425 mL). The aqueous phase is subsequently dialysed at 4°C against ultrapure water for 1-2 days (until the smell of phenol has disappeared) and finally lyophilised for 1-2 days giving the PS as a white solid. UV analysis (0.5 mg / mL) is performed as described in Example 2.

Yield: 0.262 g (23 %), UV analysis: A(260 nm): 1.52; A(280 nm): 1.09.

#### Example 8

##### SDS-PAGE analysis of PS

- 5 SDS-PAGE visualised by silver staining can be used to analyse the PS derived by hydrolysis of LPS for the presence of residual LPS. As PS do not contain the lipidic, SDS-binding lipid A moiety, PS do not complex with SDS to form charged complexes that will move during the electrophoresis. Thus, pure PS will not give rise to bands in silver-stained SDS-PAGE. The SDS-PAGE of PS preparations is performed as described in Example 3.
- 10 Figure 9 shows typical results from five different *Salmonella Choleraesuis* PS preparations. As seen from the figure only minute amounts of LPS are still present in these PS preparations.

#### Example 9

- 15 **Determination of PS ability to passively bind to micro plates**

PS derived from LPS by delipidation loses the ability to bind by passive adsorption to microplates. Thus PS preparations tested as described in Example 4 give rise to a very small OD signal as compared to a comparable amount of LPS, as seen with *Salmonella Typhimurium* and *Salmonella Choleraesuis* in Figures 7 and 8, respectively.

20

#### Example 10

##### Preparation of *Actinobacillus pleuropneumoniae* PS.

- Culture conditions. *Actinobacillus pleuropneumoniae* serotype 5b reference strain L20, and serotype 6 reference strain Femø were used for preparation of the LPS-antigen. The
- 25 strains were grown on meat broth agar plates supplemented with 5% bovine blood (Jacobsen, M.J. and Nielsen, J.P., 1995, Development of a selective and indicative medium for isolation of *Actinobacillus pleuropneumoniae* from tonsils. *Vet. Microbiol.* 47, 191-197) with a non-haemolytic *Staphylococcus aureus* as nicotinamide adenine dinucleotide (NAD) nurse-strain or on modified PPLO-agar plates (Nicolet, J., 1971,
- 30 *Zentralbl. Bacteriol. Abt. 1 Orig.* 216, 487-495, Sur l'Hemophilose du porc III. *Differentiation serologic de Haemophilus parahaemolyticus.*) as solid media. Liquid growth medium for propagation of *A. pleuropneumoniae* consisted of 30 g/l Trypticase soy broth (BBL 11768, Becton Dickinson, Cockeysville, MD, USA) supplemented with 10 g/l yeast extract (Oxoid L21, Unipath Ltd., Basingstoke, UK) and 0.03% (w/v) NAD (Sigma

Chemical Co., St. Louis, MO, USA), pH 7.2. Liquid cultures of 1 l were grown in 2 l-Erlenmeyer shaking flasks at 37°C with shaking at 130 rpm.

- Extraction. LPS was prepared from *Actinobacillus pleuropneumoniae* serotype 5b, strain L20 and serotype 6, strain Femø by a method previously described (Nielsen, R., Andresen, L.O. and Plambeck, T., 1996, Serological characterization of *Actinobacillus pleuropneumoniae* biotype 1 strains antigenically related to both serotype 2 and 7. *Acta Vet. Scand.* 37, 327-336) except that the starting material was 10 g of wet-weight cells from liquid over night cultures and volumes were scaled proportionately. The LPS was precipitated as a gel by ultracentrifugation at 90,000 x g. The precipitated LPS was dissolved in 2 mL of deionised water and freeze dried.

- Preparation of PS. PS is prepared from the freeze-dried LPS of the two *Actinobacillus pleuropneumoniae* serotypes essentially as described in Examples 6 and 7 for *Salmonella* PS. Yields of PS are expected to be in the 10-40 % range, compared to starting amount of LPS.

#### Example 11

##### UV-analysis of AQ-PS conjugates

- UV spectroscopy can be used to analyse the AQ, DNA and protein content of the PS. DNA content can be measured at 260 nm and protein content at 280 nm while the AQ content can be measured at 260 nm 280 nm and 330 nm (see "Spectroscopic methods in organic chemistry, 3'd ed., p 31", D. H. Williams and I. Fleming, McGraw-Hill (UK) 1980). Briefly, the AQ-PS is dissolved in ultrapure water to a final concentration of 0.5 mg/mL. The UV spectrum is measured from 200 - 400 nm with ultrapure water alone as the reference. Figure 10 shows a typical UV-profile of AQ-PS derived from *Salmonella* Typhimurium, while Figure 11 shows a typical UV-profile of AQ-PS derived from *Salmonella* Choleraesuis.

#### Example 12

##### Conjugation of H- $\beta$ Ala- $\beta$ Ala-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl to PS derived from *Salmonella* Typhimurium

Make up following coupling buffer and freshly prepared solutions of reagents:

- Solution 1: 1mM N-hydroxy succinimide in DMSO (6 mg N-hydroxy succinimide dissolved in 50 mL DMSO).

Solution 2: 2 mM WSC in ultrapure water (10 mg WSC dissolved in 25 mL water).

Solution 3: 2mM of reporter group in ultrapure water (for H- $\beta$ Ala- $\beta$ Ala-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl dissolve 24 mg in 25 mL water).

Buffer: Sodium hydrogen carbonate buffer, pH 8.0 (sodium hydrogen carbonate (8.4 g) is dissolved in ultrapure water and the pH adjusted to 8.0 with 1M aqueous HCl).

Weigh out PS derived from *Salmonella* Typhimurium (50 mg) into a round bottomed flask. Add solution 1 (3.13 mL) followed by the addition of solution 2 (1.56 mL), solution 3 (1.56 mL) followed by buffer (6.25 mL). The resultant clear solution is stirred over night at 4°C.

- 10 The reaction mixture is subsequently dialysed against ultrapure water for three days at 4°C, changing the ultrapure water every day, and finally lyophilised for one day to the PS-AQ conjugate as a white solid. The preparation may include AQ-LPS conjugate as well depending on the amount of intact LPS in the PS preparation. Yield: 83%; UV analysis: A (260 nm): 1.11; A (280): 0.57; A (330 nm): 0.17

15

**Example 12a:**

**Conjugation of H- $\beta$ Ala- $\beta$ Ala-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl to PS derived from *Actinobacillus pleuropneumoniae* serotype 5b.**

The solutions 1-3 are as described in Example 12.

- 20 Weigh out PS derived from *Actinobacillus pleuropneumoniae* 5b (App5b) (2 mg, 0.025 mmol - from Example 10) into a round bottomed flask. Add solution 1 (0.125 mL) followed by the addition of solution 2 (0.063 mL), solution 3 (0.063 mL) followed by buffer (0.25 mL). The resultant clear solution is stirred over night at 4°C. The reaction mixture is subsequently dialyzed against ultrapure water for three days, changing the ultrapure
- 25 water once a day. The PS-AQ solution is lyophilized for one day, whereby the PS-AQ conjugate is obtained as a white solid. The preparations may include minor amounts of AQ-LPS conjugate as well depending on the amount of intact LPS in the PS preparation.

**Example 13****Conjugation of H- $\beta$ Ala- $\beta$ Ala-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl to PS derived from *Salmonella* Choleraesuis**

Weigh out PS derived from *Salmonella* Choleraesuis (50 mg) into a round bottomed flask.

- 5 Add solution 1 (3.13 mL) followed by the addition of solution 2 (1.56 mL), solution 3 (1.56 mL) followed by buffer (6.25 mL). The resultant clear solution is stirred over night at 4°C. The reaction mixture is subsequently dialysed against ultrapure water for three days at 4°C, changing the ultrapure water every day, and finally lyophilised for one day to the PS-AQ conjugate as a white solid. The preparation may include AQ-LPS conjugate as well
- 10 depending on the amount of intact LPS in the PS preparation. Yield: 66%; UV analysis: A (260 nm): 1.29; A (280 nm): 0.58; A (330 nm): 0.15.

**Example 14****Conjugation of H- $\beta$ Ala- $\beta$ Ala-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl to PS derived from *Salmonella*****15 Infantis**

Weigh out PS derived from *Salmonella* Infantis (2 mg) into a round bottomed flask. Add solution 1 (0.125 mL) followed by the addition of solution 2 (0.063 mL), solution 3 (0.063 mL) followed by buffer (0.25 mL). The resultant clear solution is stirred over night at 4°C.

- The reaction mixture is subsequently dialysed against ultrapure water for three days at
- 20 4°C, changing the ultrapure water every day, and finally lyophilised for one day to the PS-AQ conjugate as a white solid. The preparation may include AQ-LPS conjugate as well depending on the amount of intact LPS in the PS preparation.

**Example 15****25 SDS-PAGE analysis of AQ-PS**

SDS-PAGE visualised by silver staining can be used to analyse the AQ-PS conjugate for the amount of AQ-LPS conjugate present. The AQ-PS preparations are tested by SDS-PAGE as described in Example 3. Figure 12 shows typical result from two *Salmonella* Choleraesuis AQ-PS preparations.



**Example 16****Competitive ELISA determination of immunogenic epitopes in AQ-PS and AQ-LPS**

The presence of intact immunogenic epitopes in different AQ-PS or AQ-LPS preparations can be determined by competitive ELISA on a LPS-coated microplate. Thus, the different

- 5 AQ-PS / AQ-LPS preparations are tested essentially as described in Example 5 by incubating a two-fold dilution series of the AQ-PS /AQ-LPS together with the detection antibody. The plate is treated and read as described in Example 5. The result of this analysis was that increasing amounts of AQ-PS added to the plates result in decreasing signals due to increasing competition by the added AQ-PS for the antibody. This indicated
- 10 that the antigenicity of LPS and PS is still preserved in AQ-PS.

**Example 17****Photochemical coupling of AQ-PS and AQ-LPS to polystyrene microplates. General procedure**

- 15 The lyophilised AQ-PS / AQ-LPS conjugates are dissolved in ultrapure water to a final concentration of 1 mg/mL. These stock solutions of conjugates have a long term stability (more than 3 months) when stored at 4°C protected from direct light. Prior to photocoupling the stock solutions are diluted into a 0.1 M solution of MgCl<sub>2</sub> in demineralised water. The final optimum concentration of each individual conjugate, the pH during
- 20 photocoupling and the presence of inorganic salts must be determined individually for each application (see Examples 18 and 19). The very dilute photocoupling solutions should be carefully protected against direct sunlight. The diluted solutions of conjugates are added to each well of a polystyrene microplate (100 µl/well). The plate is placed below an appropriate UV light source and subsequently irradiated with UV light for 20 minutes
- 25 Recommended UV-light sources are Philips HPA 400 (the lamp emits low energy UV-A and UV-B light mainly between 300 and 400 nm (WO 96/31557)) or Philips 25W-S fluorescent tubes that emit UV light between 310 and 400 nm with a maximum output at 345 nm. The optimum conditions for the UV-irradiation as well as irradiation time (see Example 20) must be optimised depending on the UV-light source and equipment used.
- 30 After UV-irradiation the plate is aspirated, washed with demineralised water (3 x 300 µl) and finally dried at 37°C for 30 minutes. The dry plates should be stored at room temperature in the dark.

**Example 18****Effect of AQ-PS / AQ-LPS conjugate concentration on photocoupling efficiency**

The AQ-PS / AQ-LPS from *Salmonella* Typhimurium or *Salmonella* Choleraesuis is immobilised in different concentrations onto the microplates in order to determine the efficiency of the UV irradiation to immobilise the conjugates.

Make a stock solution of AQ-PS / AQ-LPS conjugate derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis as described in Example 17 and dilute each conjugate into separate tubes to a final concentration of 1000, 750, 500, 250 and 100 ng/mL *Salmonella* Typhimurium and 1000, 750, 500, 250 and 100 ng/mL for *Salmonella* Choleraesuis. As negative control 0.1 M MgCl is used. Add the 5 individual solutions of *Salmonella* Typhimurium conjugates to column 1-2, 3-4, 5-6, 7-8 and 9-10 of a polystyrene microplate (100 µl/well) and add 0.1 M MgCl to column 11-12. Add the 5 individual solutions of *Salmonella* Choleraesuis conjugates to column 1-2, 3-4, 5-6, 7-8 and 9-10 of a polystyrene microplate (100 µl/well) and add 0.1 M MgCl to column 11-12. Place the microplates below the UV-light source and irradiate for 20 min. Following UV-irradiation aspirate the plate, wash with demineralised water (4 x 300 µl) and assay directly without drying. Add *Salmonella* Typhimurium positive reference pig sera diluted in PBS, Tween, BSA (1:400) to each well of rows A-B, *Salmonella* Typhimurium negative reference pig sera diluted in PBS, Tween, BSA (1:400) to each well of rows C-D of the first plate (100 µl/well). Add *Salmonella* Choleraesuis positive reference pig sera diluted in PBS, Tween, BSA (1:400) to each well of rows A-B, *Salmonella* Choleraesuis negative pig reference sera diluted in PBS, Tween, BSA (1:400) to each well of rows C-D, of the second plate (100 µl/well). Incubate at room temperature with gentle agitation for 60 min., aspirate and wash all wells with PBS-Tween (3 x 300 µl). Add a HRP-labelled rabbit anti-swine IgG (DAKO PO164) diluted in PBS, Tween, BSA (1:2000) to each well. Incubate for 60 minutes at room temperature with gentle agitation, wash the wells and add OPD-substrate solution to each well of the plate (100 µl/well). Incubate the plate for 20 min. in the dark and stop the enzyme reaction by the addition of 0.5 M H<sub>2</sub>SO<sub>4</sub> (100 µl/well). Read the results on a ELISA reader at 492 nm.

Figure 15 shows a typical result using a AQ-PS derived from *Salmonella* Typhimurium and Figure 16 shows a typical result using a AQ-PS derived from *Salmonella* Choleraesuis.

**Example 19****Effect of inorganic salts and pH on photocoupling efficiency of AQ-PS / AQ-LPS conjugates**

Make the following buffer and salt solutions using demineralised water: 0.1M NaCl, 0.1 M KCl, 0.1 M LiCl, 0.1 M MgCl<sub>2</sub> and PBS pH 7.2. Make a stock solution of AQ-PS conjugate derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis as described in Example 17 and dilute each conjugate into separate tubes containing the different buffer and salt solutions to a final concentration of 5 µg/mL *Salmonella* Typhimurium and 10 µg/mL for *Salmonella* Choleraesuis. As reference make two solutions of the conjugates in demineralised water as controls. Add the individual solutions of *Salmonella* Typhimurium conjugates to column 1-6 of a polystyrene microplate (100 µl/well) and the *Salmonella* Choleraesuis conjugates to column 7-12 (100 µl/well). Place the microplate below the UV-light source and irradiate for 20 min. Following UV-irradiation aspirate the plate, wash with demineralised water (4 x 300 µl) and assay directly without drying. Add *Salmonella* Typhimurium positive reference pig sera diluted in PBS, Tween, BSA (1:400) to each well of rows A1-C6, *Salmonella* Typhimurium negative reference pig sera diluted in PBS, Tween, BSA (1:400) to each well of rows D1-F6, *Salmonella* Choleraesuis positive pig reference sera diluted in PBS, Tween, BSA (1:400) to each well of rows A7-C12, *Salmonella* Choleraesuis negative reference pig sera diluted in PBS, Tween, BSA (1:400) to each well of rows D7-F12, and PBS, Tween, BSA alone in rows G and H (100 µl/well). Incubate at room temperature with gentle agitation for 60 min., aspirate and wash all wells with PBS-Tween (3 x 300µl) and add a HRP-labelled rabbit anti swine IgG (DAKO, PO164) diluted in PBS, Tween, BSA (1:2000) to each well from A1 to G12. Incubate for 60 at room temperature with gentle agitation, aspirate the wells and add OPD-substrate solution to each well of the plate (100 µl/well). Incubate the plate for 20 min. in the dark and stop the enzyme reaction by the addition of 0.5 M H<sub>2</sub>SO<sub>4</sub> (100 µl/well). Read the results on a ELISA reader at 492 nm. Figure 17 shows a typical result using a AQ-PS derived from *Salmonella* Typhimurium and Figure 18 shows a typical result using a AQ-PS derived from *Salmonella* Choleraesuis.

30

**Example 20****Effect of irradiation time on photocoupling efficiency of AQ-PS/AQ-LPS conjugates**

The AQ-PS / AQ-LPS from *Salmonella* Typhimurium or *Salmonella* Choleraesuis is immobilised onto the microplates in order to determine the UV irradiation time to immobilise the conjugates.

35

A diluted solution of AQ-PS / AQ-LPS conjugate (*Salmonella* Typhimurium and *Salmonella* Choleraesuis) diluted in 0.1M MgCl<sub>2</sub> is added to the plate. As negative control 0.1 M MgCl is added to the plate. The plate (strips) are UV irradiated for different time periods. Following UV-irradiation the plate is washed with demineralised water (4 x 300 µl) and assayed directly without drying. *Salmonella* Typhimurium positive pig sera and *Salmonella* Choleraesuis positive pig sera diluted in PBS-Tween (1:400) is added to the plate (100 µl/well). The plate is incubated at room temperature with gentle agitation for 60 min.. The plate is washed 3 times with PBST (300 µl/well). As a secondary antibody rabbit anti-mouse Ig conjugated to HRP (PO260, Dako) diluted 1:2000 in PBST is added to the plate. The plate is incubated for 1 hour with agitation. The plate is washed 3 times with PBST (300 µl/well). An OPD-H<sub>2</sub>O<sub>2</sub> solution is added to the plate (100 µl/well). The plate is incubated for 20 min in the dark. The enzyme reaction is stopped by addition of 0.5M sulphuric acid (100 µl/well). The plate is read at 492nm in an ELISA reader.

15

Figure 19 shows a typical result using an AQ-PS conjugate derived from *Salmonella* Typhimurium and an AQ-PS conjugate derived from *Salmonella* Choleraesuis.

#### Example 21

#### 20 Serological test of pig sera on photocoupled AQ-PS derived from *Salmonella* Typhimurium

PS from *Salmonella* Typhimurium conjugated to AQ is immobilised onto polystyrene microplates by the following method. The *Salmonella* Typhimurium PS-AQ conjugate is dissolved in ultrapure water. The solutions should be omitted from light. An aliquot of the *Salmonella* Typhimurium PS-AQ conjugate solution is further diluted in 0.1M MgCl<sub>2</sub> in order to obtain an optimal concentration. The diluted solution of *Salmonella* Typhimurium PS-AQ is added to the microplates (100 µL/well). The plates are irradiated in UV light for 20 minutes. After irradiation the plates are washed by the following procedure. The *Salmonella* Typhimurium PS-AQ conjugate solution is discharged and 300 µL of water is added using a washer. The microplates are subsequently washed 3 times in water (3x300µL). The microplates are dried at 37°C for 30 minutes. The microplates are allowed to equilibrate to room temperature for 15 minutes before packing or further use. The plates should be stored at room temperature. For serological use sera and/or meat juice samples from swine are investigated. The ELISA procedure used is an indirect

35 ELISA as followed. An appropriate panel of reference sera is tested on each plate parallel

to the specimen samples. Specimen samples or reference sera (100 µL/well) diluted in PBS, Tween, BSA, are added to each well. The plate is incubated for one hour with gentle agitation. The plate is washed in PBS, Tween (3x300 µL). Secondary antibody conjugated to HRP (rabbit anti-swine IgG HRP, PO164, Dako) (100 µL/well) diluted in PBS, Tween, BSA, is added to each well. The plate is incubated for one hour with gentle agitation. The plate is washed in PBS, Tween (3x300 µL). OPD solution (100 µL/well) is added to the plate. The plate is incubated for 20-25 minutes, depending on how fast the controls reaches the appropriate OD values. The enzyme reaction is stopped by addition of 100 µL of 0.5 M sulphuric acid. The plate is shaken manually and read at 492 nm on an ELISA reader.

The results are shown in Figure 20. As seen in the figure sera 1-5 are *Salmonella* negative while sera 6-15 are positive for *Salmonella* Typhimurium.

#### 15 Example 24

##### **Serological tests using covalently coupled lipopolysaccharide polysaccharides: A mixture of coupled *Salmonella* polysaccharides.**

In a comparative experiment plates were coated with a mixture of *Salmonella* Typhimurium and *Salmonella* Choleraesuis polysaccharides as described above (covalent mix-ELISA) and compared to plates conventionally coated ("Mix-ELISA" (Nielsen 1995)) with whole LPS from the same two *Salmonella* serotypes. Four *Salmonella* Typhimurium positive, two *Salmonella* Infantis positive and one *Salmonella*- negative sera were used as reference sera to calibrate the OD-readings from each ELISA plate, exactly as described previously (Nielsen 1995), see below. The covalent Mix-ELISA was performed as the previously published mix-ELISA protocol (Nielsen et al. 1995), except that the blocking step (with bovine serum albumin, BSA) was omitted. Sera were diluted 1:400 in PBS, Tween, BSA. Duplicates of 100 µl of each serum was applied and incubated for 1 h at room temperature without agitation. The plates were then washed three times in PBS, T and subsequently incubated with HRP-conjugated rabbit anti swine IgG (PO164, DAKO) diluted 1:3000 in PBS, Tween, BSA for 1h at room temperature. The plates were then washed as before, and 100 µL OPD substrate solution was added to each well and incubated 10-15 min. The reaction was stopped with 100 µL H<sub>2</sub>SO<sub>4</sub> and the optical density was read at 490 nm, subtracting 650 nm (background correction).

To test the AQ-PS-coated plates a panel of 20 sera with known reactivity in the mix-ELISA system were used (20 sera from pigs from multiplying herds). A typical result is shown in Figure 21. When using a cut-off at 10 OD%, all of the sera remained in the same group of positives or negatives, when being tested in the AQ-PS-ELISA and the mix-ELISA, respectively. The OD-level of the low responding samples correlated well between the two systems. For the positive samples a slight reduction was observed in the AQ PS-ELISA for some of the samples, but this feature varied from day to day.

#### Example 25

##### 10 Reproducibility of photocoupled mixed AQ-PS derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis

The reproducibility of the AQ-PS immobilised microplates has been investigated over a longer period. Microplates have been immobilised with mixed AQ-PS derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis as described above. Reference sera have been added to the plates in 16 duplicates on each plate. This procedure have been repeated on four plates each day over a period of four days. The results were normalised against a control reference serum. Intraplate variation was measured to 7.7% while interplate variation was measured to 5.1%

#### 20 Example 26

##### Storage stability of photocoupled mixed AQ-PS derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis

The storage stability of the *Salmonella* Typhimurium and *Salmonella* Choleraesuis mixed AQ-PS ELISA plates is a important factor to investigate. A long storage stability provides a plate that is independent of coating procedures prior to use and provides a plate that shows minimal variations within the same batch as well as between batches. The stability of the PS-AQ immobilised microplates has been investigated over a period of several months. Plates were prepared as described in Example 23 and stored at room temperature in the dark and then the plates were tested using a panel of reference sera directed against *Salmonella* Typhimurium and *Salmonella* Choleraesuis O-antigens in PS/LPS over a period of 71 days. The results are seen in Figure 22. As seen in Figure 22, the plates were stable for at least 71 days.

**Example 27****Detection of infection levels of swine herds using a serological test based on photocoupled mixed AQ-PS derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis**

5 The microplates photocoupled with mixed AQ-PS derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis have been investigated for serological use. For serological surveillance of swine herds it is of great importance to achieve a standardised microplate with minimal variations in order to achieve a reliable and reproducible response in each assay. To obtain this, one has to produce plates with the previously described qualities.

10

A panel of sera from 59 conventional swine herds (10 sera from each herd) was investigated using a photocoupled mixture of *Salmonella* Typhimurium and *Salmonella* Choleraesuis PS as described in Example 26. The result of this analysis was compared to the result of the analysis of the same samples in a conventionally coated "Mix-ELISA" (Nielsen 1995) using

15 LPS from the same two types of *Salmonella*. The herds were classified as having a low prevalence of *Salmonella* based on the Mix-ELISA and based on the principles of the Danish Salmonella surveillance program (Mousing J, Jensen PT, Halgaard C, Bager F, Feld N, Nielsen B, Nielsen JP, Bech-Nielsen S: 1997, Nation-wide *Salmonella* enterica surveillance and control in Danish slaughter swine herds. Prev. Vet. Med. 29: 247-261.)

20

The ELISA procedure used is an indirect ELISA as followed. An appropriate panel of reference sera are tested on each plate parallel to the specimen samples. Specimen samples or reference sera (100 µL/well) diluted in PBS, Tween, BSA, are added to each well. The plate is incubated for one hour (the use of agitation during incubation may

25 enhance the P/N ratio between positive and negative samples). The plate is washed in PBS, Tween (3x300 µL). Secondary antibody conjugated to HRP (rabbit anti-swine IgG HRP, PO164, Dako) (100 µL/well) diluted in PBS, Tween, BSA (1:2000) is added to each well. The plate is incubated for one hour with mild agitation. The plate is washed in PBS, Tween (3x300 µL). OPD solution (100 µL/well) is added to the plate. The plate is

30 incubated for 20-25 minutes, depending on how fast the controls reaches the appropriate OD values. The enzyme reaction is stopped by addition of 100 µL of 0.5 M sulphuric acid. The plate is shaken manually and read at 492 nm on an ELISA reader.

Only four out of the 590 samples were positive in the photocoupled assay, maintaining the classification of all herds as having a low *Salmonella* prevalence as also found with the conventional "mix-ELISA".

## 5 Example 28

### Detection of *Salmonella* Typhimurium bacteria using a competitive ELISA test based on photocoupled mixed AQ-PS derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis

The microplates photocoupled with mixed AQ-PS derived from *Salmonella* Typhimurium and *Salmonella* Choleraesuis can be used as an antigen ELISA for detection of *Salmonella* bacteria or *Salmonella* antigen. Detection of *Salmonella* bacteria in foods or environmental has a great potential. Detection of *Salmonella* bacteria can be accomplished by use of the photocoupled mixed AQ-PS *Salmonella* Typhimurium and *Salmonella* Choleraesuis as a competitive ELISA by the following procedure.

15

Bacteria samples are added to the plates in two fold dilutions, diluted in PBS (50  $\mu$ L/well). As a control PBS is added (without any bacteria samples) to the plates (50  $\mu$ L/well). A monoclonal antibody (50  $\mu$ L/well) diluted in PBS, Tween, BSA, directed against the relevant *Salmonella* serotype is added to the plate. As a negative control the monoclonal antibody is added to a well with PBS. As a positive control PBS, Tween, BSA is added to a well with PBS. The plate is incubated for one hour with gentle agitation. The plate is washed in PBS, Tween (3x300  $\mu$ L). Secondary antibody conjugated to HRP (rabbit anti-mouse IgG HRP, PO260, Dako) (100  $\mu$ L/well) diluted in PBS, Tween, BSA, is added to each well. The plate is incubated for one hour using agitation. The plate is washed in PBS, Tween (3x300  $\mu$ L), make sure that all wells are filled during each wash. OPD solution (100  $\mu$ L/well) is added to the plate. The plate is incubated for 20-25 minutes, depending on how fast the controls reaches the appropriate OD values. The enzyme reaction is stopped by addition of 100  $\mu$ L of 0.5 M sulphuric acid. The plate is shaken manually and read at 492 nm on an ELISA reader. The wells with monoclonal antibody (are calculated as 100% response). The wells with PBS, Tween, BSA (are calculated as 0% response). The OD response of the wells with bacteria samples are calculated in relation to the positive and the negative controls. The decrease of the response is proportional to the increase in positivity of the sample. From the titration curve the titre is calculated. The titre is the dilution factor of the bacterial solution leading to a 50% decrease



in the OD-value compared to the OD-value obtained without competing antigen (100% response).

### Example 29

#### 5 Conjugation of biotin to PS derived from *Salmonella* Typhimurium

Make up following coupling buffer and freshly prepared solutions of reagents:

Solution 1: 1 mM N-hydroxy succinimide in DMSO (6 mg N-hydroxy succinimide dissolved in 50 mL DMSO).

Solution 2: 2 mM WSC in ultrapure water (9.6 mg WSC dissolved in 25 mL water).

10 Solution 3: 2 mM of biotin-amine in ultrapure water (dissolve 19 mg in 25 mL water).

Buffer: Sodium hydrogen carbonate buffer, pH 8.0 (sodium hydrogen carbonate (8.4 g) is dissolved in ultrapure water and the pH adjusted to 8.0 with 1M aqueous HCl).

The resultant clear solution is stirred overnight at 4°C. The reaction mixture is

15 subsequently dialysed against ultrapure water for three days, changing the ultrapure water every day. The PS-biotin conjugate is finally lyophilised overnight. The lyophilised PS-biotin conjugate is dissolved in ultrapure water to a final concentration of 1 mg/mL. This stock solution of PS-biotin conjugate have a long term stability (more than 3 months) when stored at 4°C protected from direct light. The stock solutions are diluted in PBS pH

20 7.2, 1:100 and 1:400. The diluted PS-biotin solutions are added to a microplate (100 µl/well) coated with streptavidin (Pierce). As a control diluted PS solutions (1:100 and 1:400 in PBS) are added to the microplate (100 µl/well). The plate is incubated for 1 hour with agitation. The plate is washed 3 times with PBST (300 µl/well). A monoclonal antibody against *Salmonella* Typhimurium LPS (O-chain) (S9, HyTest Ltd.) is added to the

25 plate in a two-fold dilution (diluted in PBST) 100 µl/well. The plate is incubated for 1 hour with agitation. The plate is washed 3 times with PBST (300 µl/well). As a secondary antibody rabbit anti-mouse Ig conjugated to HRP (PO260, Dako) diluted 1:2000 in PBST is added to the plate. The plate is incubated for 1 hour with agitation. The plate is washed 3 times with PBST (300 µl/well). An OPD-H<sub>2</sub>O<sub>2</sub> solution is added to the plate (100 µl/well).

30 The plate is incubated for 20 min in the dark. The enzyme reaction is stopped by addition of 0.5M sulphuric acid (100 µl/well). The plate is read at 492nm in an ELISA reader. The results are seen in Figure 23. As seen in Figure 23 only the biotin conjugated PS binds to the streptavidin plate.

**Example 30****Clinical tests using covalently coupled lipopolysaccharide polysaccharides:****Coupled *Actinobacillus pleuropneumoniae* serotype 5b polysaccharides.**

Polysaccharides derived from *Actinobacillus pleuropneumoniae* serotype 5b (App5b) as described above (Example 10) are derivatised with AQ as described for *Salmonella* PS in Example 12a, and subsequently coupled to PolySorp immunoplates from Nunc as described above (Example 17). The microtiter plates were covalently coupled with the APP 5b PS-AQ conjugate in a two-fold dilution from 500 ng to 0.98 ng PS-AQ per well. A positive and a negative serum for APP 5b diluted 1:400 in PBS-T was applied to the plates. The plates were incubated for 1 hour. The plates were washed 3 times in PBS-T and subsequently incubated with a secondary antibody (rabbit anti-swine Ig HRP-conjugated). The plates were incubated for 1 hour and subsequently washed 3 times in PBS-T. The plates were developed with OPD.

The results are shown in Figure 24. The Figure shows that the *Actinobacillus pleuropneumoniae* serotype 5b lipopolysaccharide derived polysaccharide can be conjugated to AQ and covalently coupled to a microplate surface. The antigenicity was retained as the PS-AQ antigen coated plates showed similar response as to LPS passively coated plates. *Actinobacillus* is a Gram-negative bacteria of the HAP family and is therefore taxonomically not closely related to *Salmonella* strains. This indicates that LPS from Gram-negative bacteria in general may be successfully converted to PS-AQ conjugates.

**Example 31****Clinical tests using covalently coupled lipopolysaccharide polysaccharides:****Coupled *Actinobacillus pleuropneumoniae* serotype 6 polysaccharides**

Polysaccharides derived from *Actinobacillus pleuropneumoniae* serotype 6 as described above (Example 10) are derivatised with AQ as described for *Salmonella* PS in Examples 12-13 and subsequently coupled to PolySorp immunoplates from Nunc as described above (Example 17). This surface is compared to a conventional surface prepared by adsorbing whole LPS from the same *Actinobacillus pleuropneumoniae* (Ap) strain in a competition ELISA using a polyclonal antibody as detection antibody and blockade by well-defined pig sera. Briefly, the ELISA is performed as follows: The optimal concentration of the Ap6 LPS antigen, serum and enzyme conjugate are determined by checkerboard titrations in ELISA plates. The microplates are coated with LPS diluted

- 1:10.000 in PBS and incubated overnight at 4°C. The plates are blocked with PBS with 0.05% Tween 20 and with 1% bovine serum albumin (PBS, T, BSA) for 30 min at room temperature (RT). Test sera are diluted 1:10 in PBS, T, BSA and added to the wells in duplicate and incubated for 1 h at RT. Without emptying the plates rabbit serum diluted
- 5 1:8000 is added for 30 min at RT. Afterwards the plates are incubated with peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako, Copenhagen, Denmark) diluted 1:1000 in PBS, T, BSA. The substrate-dye solution o-phenylenediamine-H<sub>2</sub>O<sub>2</sub> is allowed to react for 15 min and the reaction is stopped by the addition of 0.5 M H<sub>2</sub>SO<sub>4</sub>. The optical density at 490 nm is measured with 650 nm as the reference. The optical density of the
- 10 rows containing PBS, T, BSA without serum is used for calculation of the percent inhibition by the individual sera. The dilution and incubation time for the test serum and the rabbit serum are adjusted to give 0-25% inhibition with negative sera and 80-100% with positive serum.
- 15 The results are expected to show that the Ap-derived polysaccharide can be coupled covalently to the microplate surface while retaining its antigenicity and showing a blocking behaviour comparable to or better than conventionally non-covalently coated whole LPS.

### Example 32

#### 20 Conjugation of H<sub>2</sub>N-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl to KDO

- KDO (10 mg, 40 μmol), H<sub>2</sub>N-(CH<sub>2</sub>)<sub>3</sub>-NHCO-AQ · HCl (7 mg, 20 μmol) and BOP (21 mg, 48 μmol) was dissolved in DMSO. Then, triethyl amine (17 μl, 120 μmol) was added and the clear solution stirred at room temperature for 60 hours. Water (40 mL) was added to the reaction mixture followed by freeze drying. The solid product mixture was
- 25 redissolved/resuspended in water (1 mL) and added to a SeppPak C18 column (1 mL) that had previously been equilibrated with acetonitrile (7 mL) and water (7 mL). The column was eluted with water (10 mL) and then water/acetonitrile (10 mL, 1:1 V/V). The first aqueous fraction contained mainly excess KDO and other impurities, while the second water/acetonitrile fraction contained the AQ-KDO conjugate. TLC (1-butanol /
- 30 acetic acid / water 4:1:1): R<sub>f</sub> = 0.33. MS (MALDI-TOF): 545.8 (MH<sup>+</sup> + H<sub>2</sub>O).

**Example 33**

**A method for estimating the number of a specific bacteria, serotyping of bacteria or antigen (LPS/PS) detection is established by use of AQ-PS immobilised microplates.**

- 5 A solution of bacteria or an antigen solution (LPS/PS) is added to the plates in a two fold dilution (50µL/well). A monoclonal antibody against *Salmonella* Typhimurium or *Salmonella* Choleraesuis diluted in PBS, Tween, BSA (50 µL /well) is added to the plate. The plate is incubated for one hour with mild agitation. The plate is washed 3 times with PBS, Tween (300µL/well). A rabbit anti-mouse IgG conjugated to HRP (PO260, Dako)
- 10 diluted in PBS, Tween, BSA (100µL /well) is added to the plate. The plate is incubated for one hour with mild agitation. The plate is washed 3 times with PBS, Tween (300 µL/well). An OPD-solution is added to the plate (100 µL /well). The plate is incubated for 20 min in the dark. The reaction is stopped by addition of 0.5M H<sub>2</sub>SO<sub>4</sub> (100 µl/well). The plate is read at 492 nm in an ELISA reader. This method provides the detection of small amounts
- 15 of bacteria in biological fluids and provides the determination of a bacteria serotype.

## CLAIMS

1. A method for immobilising a polysaccharide (PS) to a solid surface, said polysaccharide having a keto-carboxy group ( $-C(=O)-COOH$ ) or a ketal or hemiketal group corresponding thereto), the method comprising the steps of:

- a) forming a covalent bond between the carboxy group of the polysaccharide and a reporter molecule (RM), thereby forming a polysaccharide-reporter molecule conjugate (PS-RM), said reporter molecule comprising a recognition/substrate site; and
- b) immobilising the polysaccharide-reporter molecule conjugate (PS-RM) by forming a specific bond between the recognition/substrate site of said reporter molecule and a reception/reagent site of the solid surface.

2. A method for immobilising a polysaccharide (PS) to a solid surface, said polysaccharide having a keto-carboxy group ( $-C(=O)-COOH$ ) or a ketal or hemiketal group corresponding thereto), by forming a covalent bond between the carboxy group of the polysaccharide and a chemical functionality linked to the solid surface.

3. A method according to claim 1 or 2, wherein the keto-carboxy group of the polysaccharide is a part of a KDO (2-keto-3-deoxy-D-manno-octonic acid) monosaccharide unit of the polysaccharide.

4. A method according to any of the preceding claims, wherein the polysaccharide (PS) is substantially identical to the carbohydrate part of a Gram-negative bacterial lipopolysaccharide (LPS).

5. A method according to claim 4, wherein the polysaccharide is obtained by selective hydrolysis of the ketal bond between the inner core part and the lipid A part of a Gram-negative bacterial lipopolysaccharide.

6. A method according to claim 1 or 3, wherein the polysaccharide further comprises the lipid part of a Gram-negative bacterial lipopolysaccharide, thereby constituting a bacterial lipopolysaccharide.

7. A method according to any of the claims 4-6, wherein the bacterial lipopolysaccharide is derived from a bacteria selected from Gram-negative bacteria that are human or veterinary pathogens, e.g. enterobacteria, respiratory bacteria, urogenital bacteria, and neuropathogenic bacteria.

5

8. A method according to claim 7, wherein the bacteria is a veterinary pathogen or zoonotic bacteria including enterobacteria selected from *Escherichia coli*, *Salmonella* Typhimurium, *Salmonella* Choleraesuis, *Salmonella enterica*, and all serotypes hereof, especially *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Choleraesuis, *Salmonella*

10 Manhattan, *Salmonella* Dublin, *Salmonella* Infantis, *Escherichia coli* spp. including O157, oedema-disease causing *Escherichia coli*, *Yersinia enterocolitica*, and *Campylobacter jejuni*.

9. A method according to claim 7, wherein the bacteria is a respiratory bacteria selected from the HAP group of bacteria especially *Actinobacillus* sp; in particular the HAP bacteria *Actinobacillus pleuropneumoniae*, *Haemophilus somnus*, *Pasteurella haemolytica*, *Pasteurella multocida*, *Haemophilus parasuis* and *Mannheimia* sp.

10. A method according to claim 1 or 2, wherein the polysaccharide has a molecular weight of at least 1000.

11. A method according to any of the claims 1 and 3-10, wherein formation of the covalent bond between the polysaccharide and the reporter molecule is mediated by using a coupling reagent.

25

12. A method according to any of the claims 2-10, wherein formation the covalent bond between the polysaccharide and the chemical functionality of the solid surface is mediated by using a coupling agent.

13. A method according to any of the claims 1 and 3-11, wherein the reporter molecule comprises a reporter part for presenting the recognition/substrate site and a linker part for linking the reporter part to the polysaccharide.

14. A method according to claim 13, wherein the polysaccharide-reporter molecule conjugate (PS-RM) has the general formula  $PS'-C(=O)-N(R^N)-F-L-R$ , where  $PS'-C(=O)$  is

the polysaccharide,  $N(R^N)-F$  is the group directly involved in the covalent link between the polysaccharide and the reporter molecule,  $R^N$  designates hydrogen or  $C_{1-4}$ -alkyl, L is the linker part of the reporter molecule, and R is the reporter part of the reporter molecule.

5 15. A method according to claim 14, wherein -N-F- designates amino (-N-), anilino (-N-Ph), hydrazido (-N-C(=O)-), semicarbazido (-N-C(=O)-NH-), thiosemicarbazido (-N-C(=S)-NH-), or hydrazino (-N-NH-), preferably amino.

10 16. A method according to claim 14, wherein L designates a biradical selected from  $C_{1-20}$ -alkylene optionally comprising aromatic or mono-/polyunsaturated hydrocarbons or cyclic hydrocarbons, oligo-oxyethylenes, oligo-amides such as oligo-glycine, oligo-alanine, oligo-lysine and oligopeptides in general, oligo-phosphodiester, oligo-phosphoamidates, oligo-phosphodiamides, oligo-sulfonesters, and oligo-sulfonamides.

15 17. A method according to any of the claims 1 and 3-16, wherein the reporter molecule has a molecular weight of at the most 10,000, preferably at the most 5,000, in particular at the most 2,500.

20 18. A method according to claim 14, wherein the reporter part is a photochemically reactive group.

19. A method according to claim 14, wherein the reporter part is a thermochemically reactive group.

25 20. A method according to claim 14, wherein the reporter part is a one part of an affinity pair.

30 21. A method according to any of the preceding claims, wherein the solid surface is a surface of an organic polymer, a glass, silicium, silicium oxide (silica) or a composite material thereof.

22. A method according to any of the preceding claims, wherein two or more types of polysaccharides are immobilised, where such types represent different bacterial serotypes.

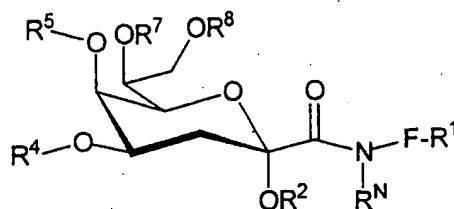
35

23. A method according to any of the claims 4-22, wherein the LPS from which the PS is derived is LPS from a *Salmonella* bacteria and the reporter molecule comprises a reporter part selected from anthraquinones and biotin, preferably anthraquinone.
- 5 24. A method according to any of the claims 4-22, wherein the LPS from which the PS is derived is LPS from an *Actinobacillus* bacteria and the reporter molecule comprises a reporter part selected from anthraquinones and biotin, preferably anthraquinone.
25. A solid surface obtainable according to the method defined in any of the claims 1-24.
- 10 26. The use of a solid surface as defined in claim 25 in a diagnostic assay.
27. The use according to claim 26, wherein the diagnostic assay is a solid phase immunoassay.
- 15 28. The use according to claim 26, wherein the diagnostic assay is a serological assay, e.g. a serological assay for detection of antibodies against microorganisms.
29. The use according to claim 26, wherein the diagnostic assay is a serological assay for
- 20 detection of antibodies against *Salmonella* spp.
30. The use according to claim 26, wherein the diagnostic assay is a serological assay for detection of antibodies against *Actinobacillus* spp.
- 25 31. An assay device for the detection of antibodies against one or more Gram-negative bacteria, comprising a solid surface having immobilised thereto a polysaccharide (PS), said polysaccharide (PS) corresponding to the carbohydrate part of the bacterial lipopolysaccharide (LPS) of the Gram-negative bacteria, via the carboxylic acid group of a KDO monosaccharide unit of said polysaccharide.
- 30 32. An assay device according to claim 31, wherein the polysaccharide is immobilised to the solid surface according to the method defined in any of the claims 1-24.



33. A method for estimating the number of bacteria or serotyping of bacterial antigen in a sample comprising whole bacteria, parts or lysates thereof, wherein an assay device according to claim 31 or 32 is used.

5 34. A compound of the general formula I



(or, in the case where R² is hydrogen, optionally the keto analogue thereof)

wherein

R¹ is selected from hydrogen and a reporter molecule L-R, where L is an optional linker

10 part of the reporter molecule, and R is a report part of the reporter molecule;

Rᴺ is selected from hydrogen and C₁-₄-alkyl;

F is selected from a single bond, phenylene, carbonyl (C(=O)), carbonylimino (C(=O)-NH-), thiocarbonyl ((C(=S)) and imino (-NH-);

R², R⁷, R⁸ are each independently selected from hydrogen and hydroxy protecting groups;

15 R⁴ is selected from hydrogen, a mono- or disaccharide residue and a hydroxy protecting group; and

R⁵ is selected from hydrogen, an "optionally functional group protected polysaccharide residue" and a hydroxy protecting group.

20 35. A compound according to claim 34, wherein R¹ is hydrogen.

36. A compound according to claim 34, wherein R¹ is L-R.

37. A compound according to claim 34, wherein -N-F- designates amino (-N-), anilino (-N-Ph), hydrazido (-N-C(=O)-), semicarbazido (-N-C(=O)-NH-), thiosemicarbazido (-N-C(=S)-NH-), or hydrazino (-N-NH-), preferably amino.

38. A compound according to claim 34, wherein L designates a biradical as defined in claim 16.

30

39. A compound according to claim 36, wherein the reporter molecule (R) has a molecular weight of at the most 10,000, preferably at the most 5,000 and most preferred at the most 2,500.

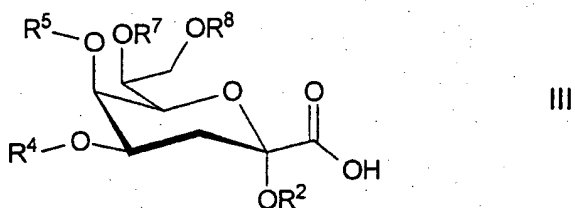
5 40. A compound according to claim 34, wherein  $R^4$  is selected from KDO-2-yl, 4-phosphoethanolamine-KDO-2-yl, L-rhamnosyl-(1→4)-KDO-2-yl, and KDO-(2→4)-KDO-2-yl.

41. A compound according to claim 34, wherein the polysaccharide residue preferably  
10 comprises from 2-997 linked monosaccharide units, such as at least 7, e.g. 7-997, linked monosaccharide units, in particular at least 22, e.g. 22-497, linked monosaccharide units, such polysaccharide residue optionally being non-stoichiometrically substituted.

42. The use of a compound according to claim 34 for the preparation of an assay device  
15 for the detection of antibodies against Gram-negative bacteria.

43. The use of a compound according to claim 34 for the preparation of a solid surface carrying an immobilised polysaccharide.

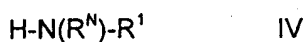
20 44. A method for the preparation of a compound of the general formula I as defined in claim 34, comprising reacting a compound of the general formula III



wherein  $R^2$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^7$  and  $R^8$  are as defined in claim 34,

25

with a nitrogen compound of the general formula IV



30 wherein  $R^1$  and  $R^N$  are as defined in claim 34;

and optionally fully or partly deprotecting the product thereby obtained in order to obtain a compound of the general formula I.

5 45. A method according to claim 44, wherein the compound of the general formula III is used in its activated ester form.

46. A method according to claim 44, wherein the reaction between the carboxylic acid  
compound of the general formula III and the nitrogen compound IV is facilitated by using a  
10 coupling reagent.

1/24

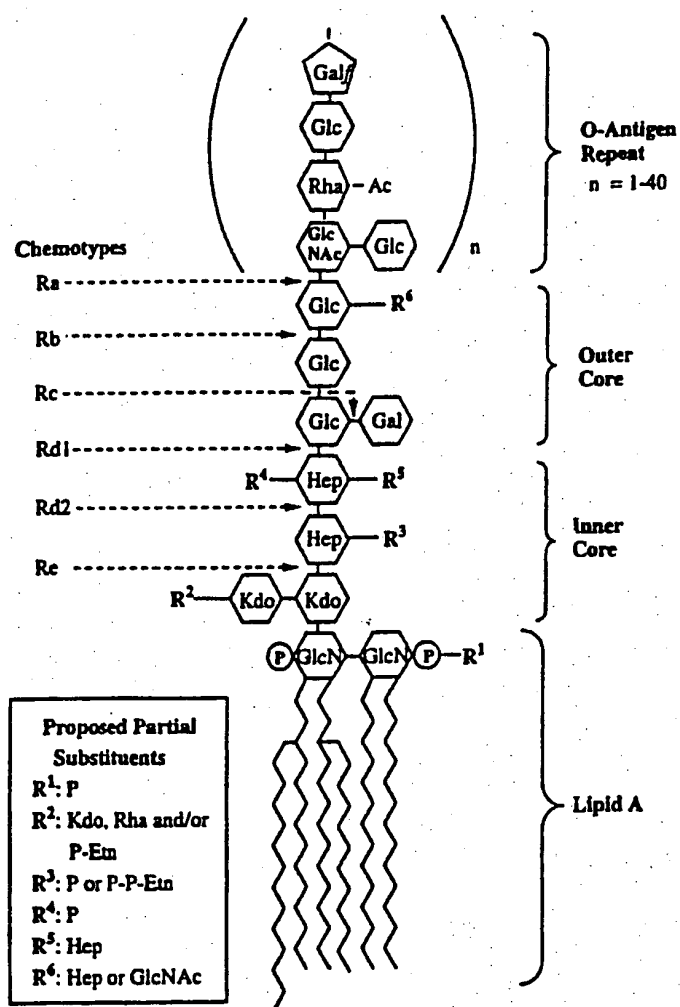


Fig. 1

2/24

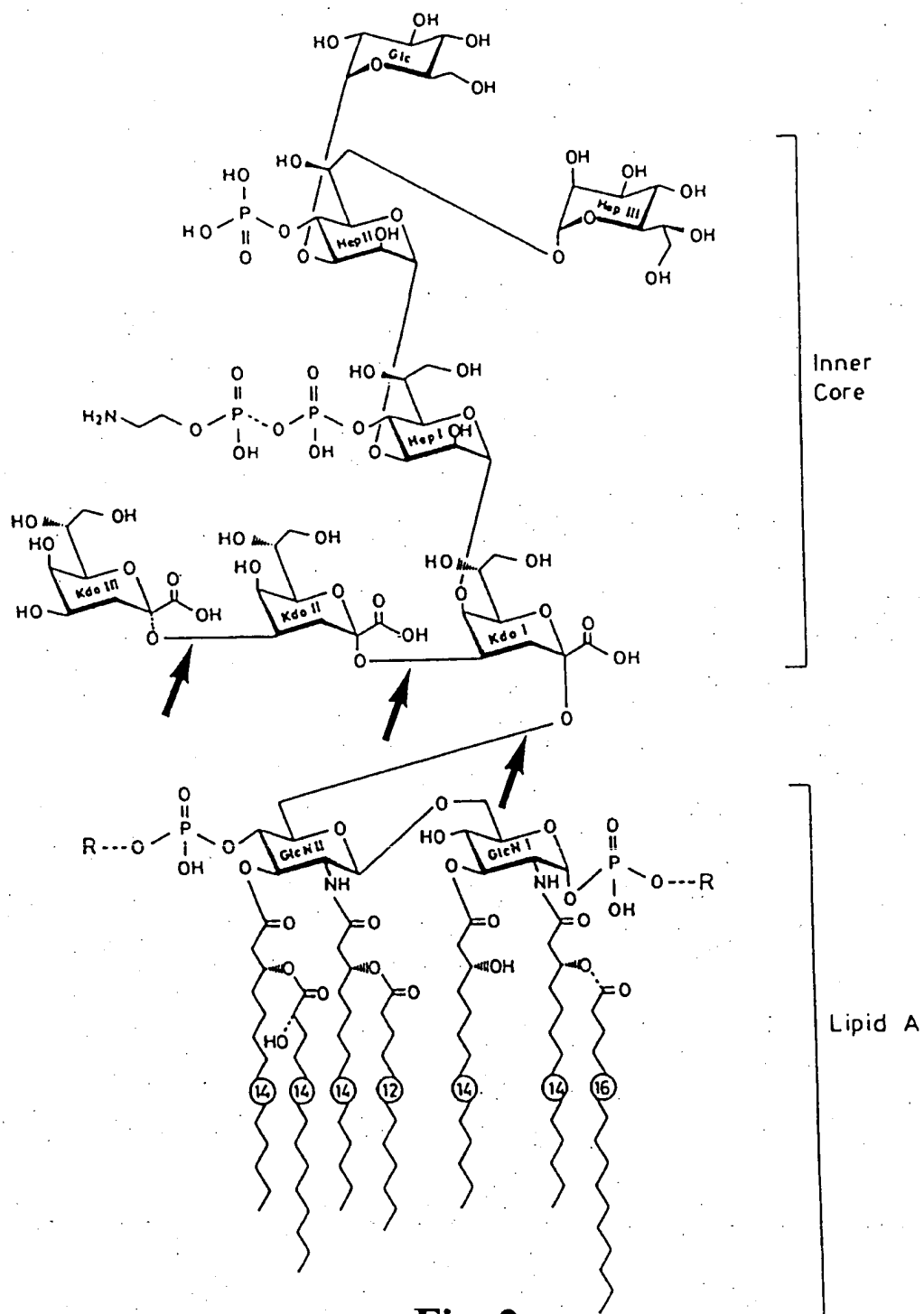


Fig. 2

3/24

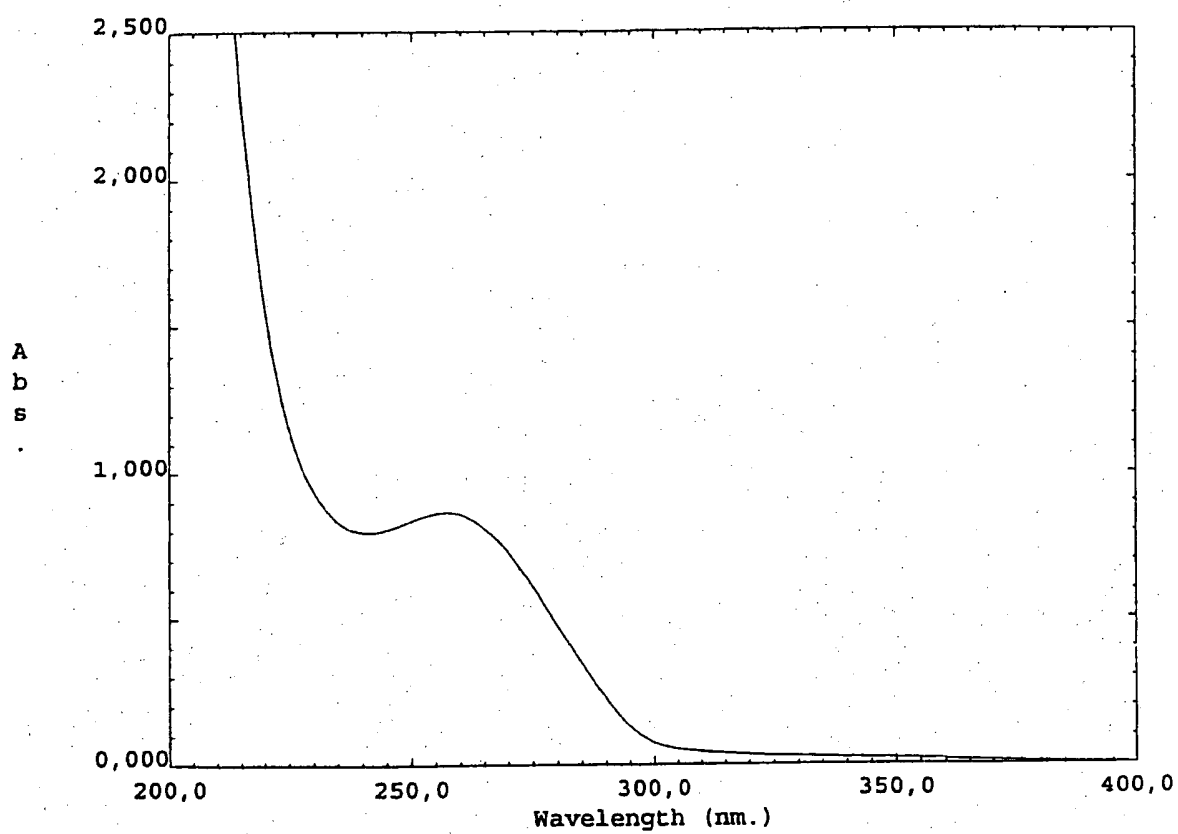


Fig. 3

4/24

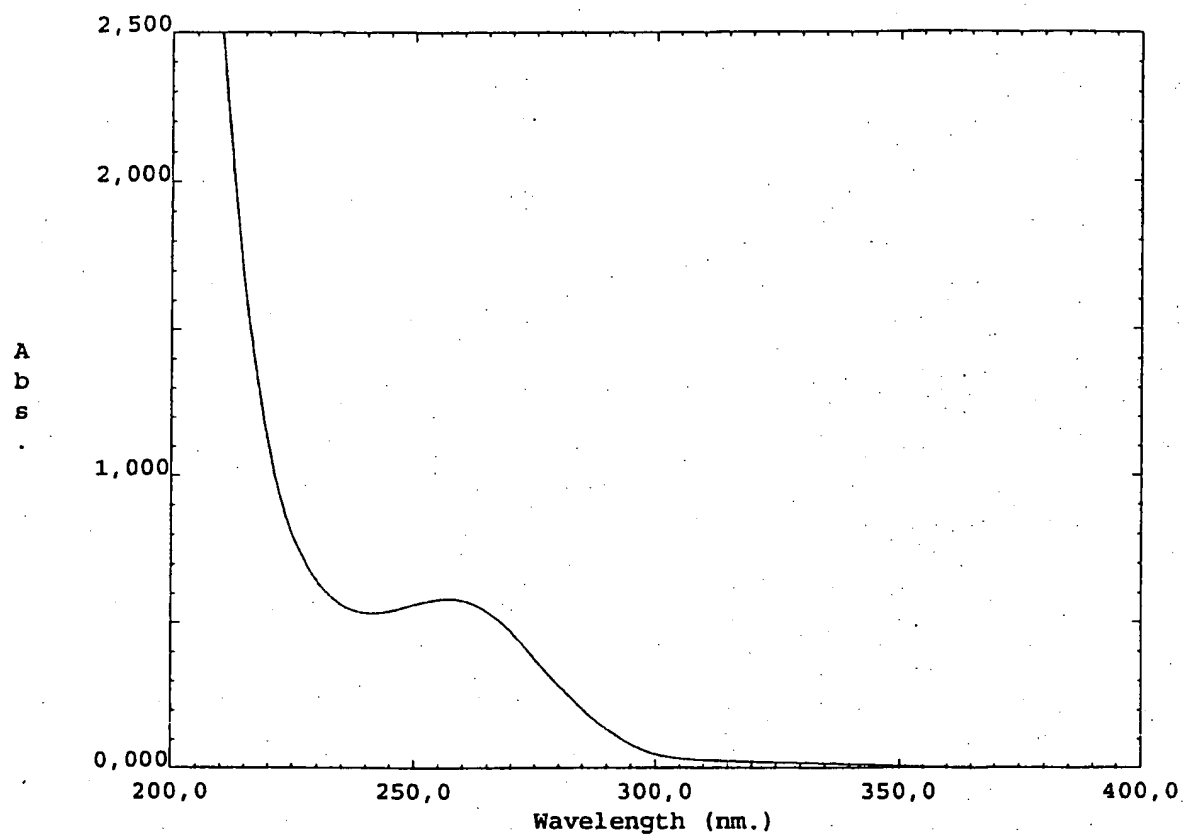


Fig. 4

5/24

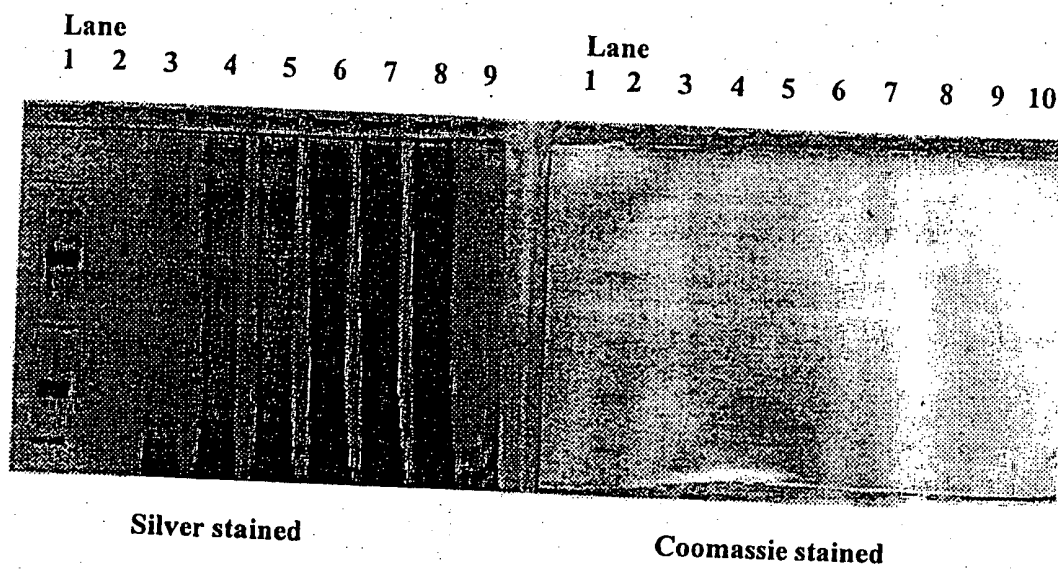


Fig. 5



6/24

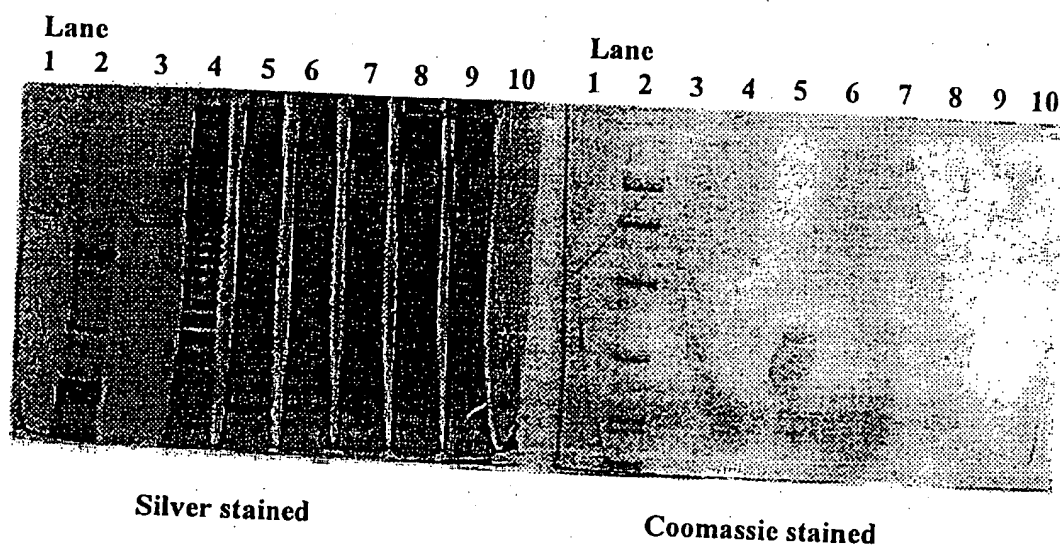


Fig. 6

7/24

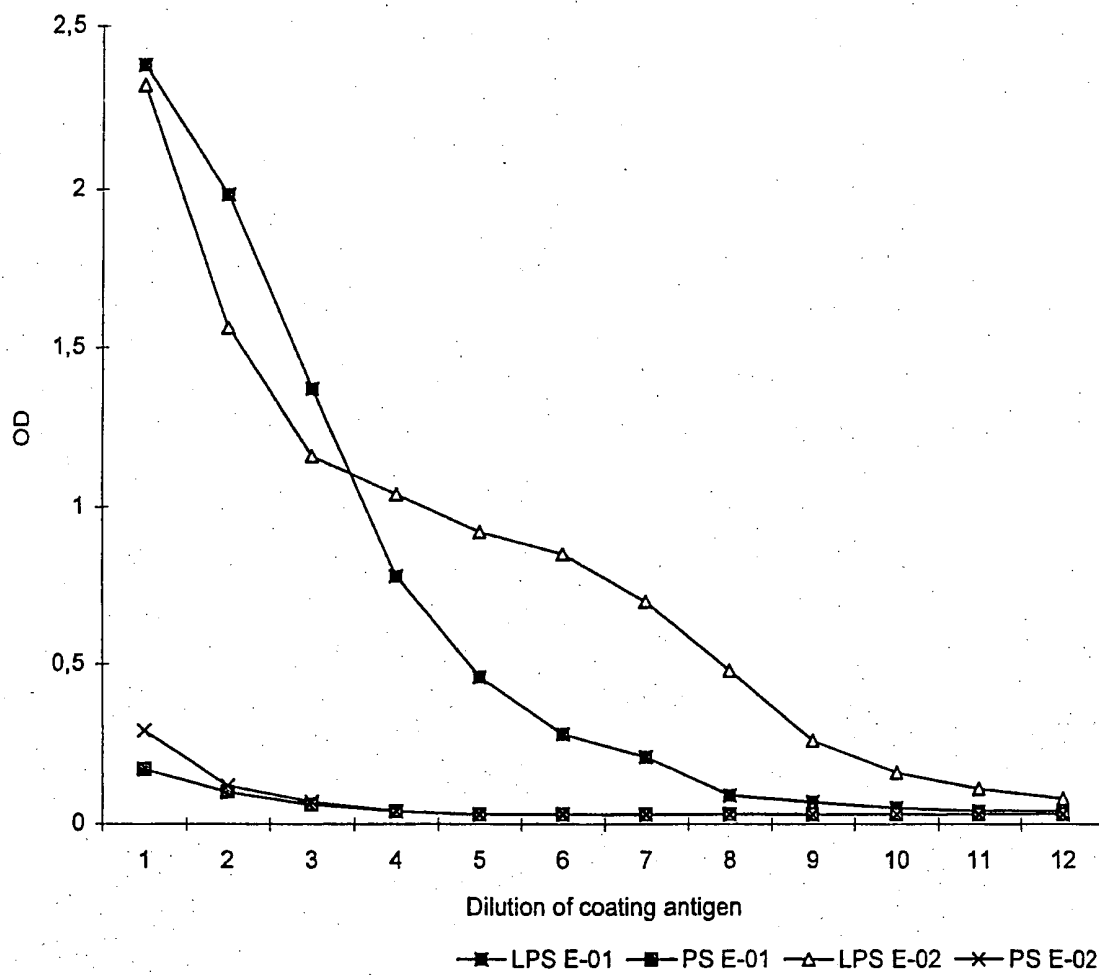


Fig. 7

8/24

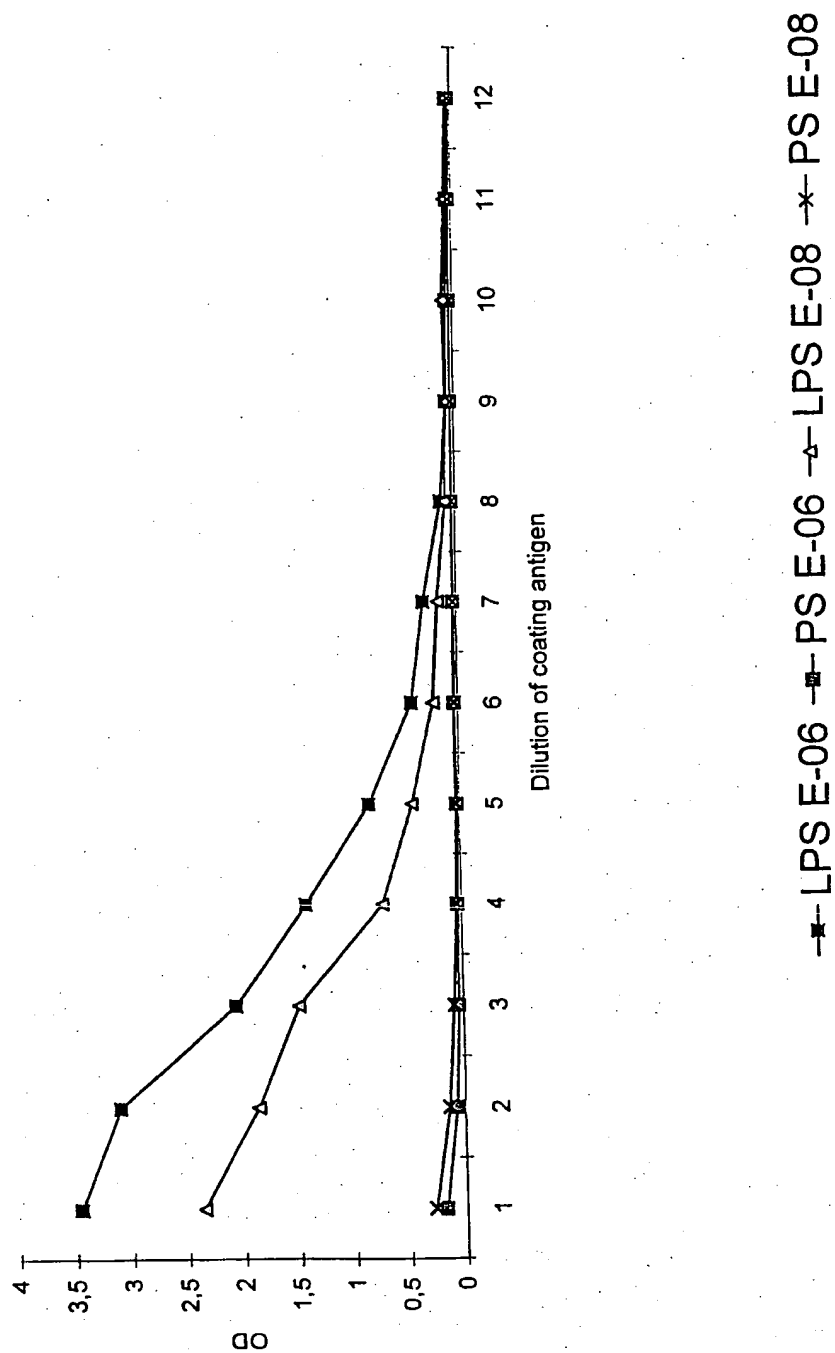


Fig. 8

9/24

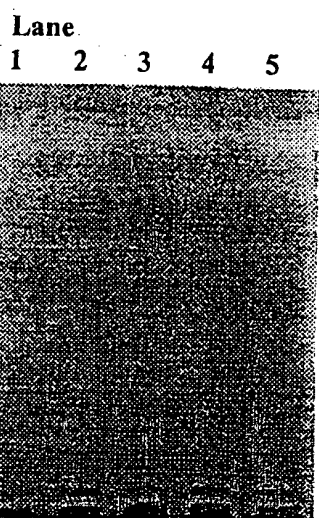


Fig. 9

10/24

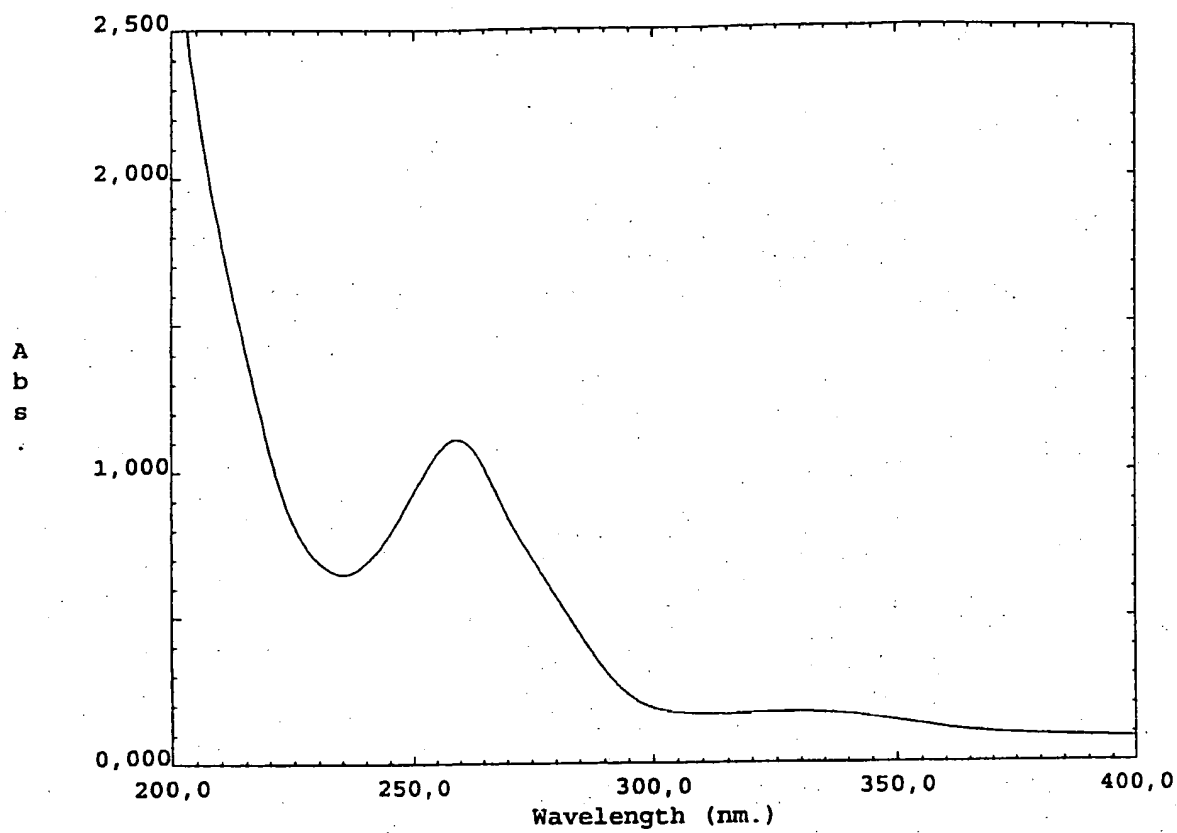


Fig. 10

11/24

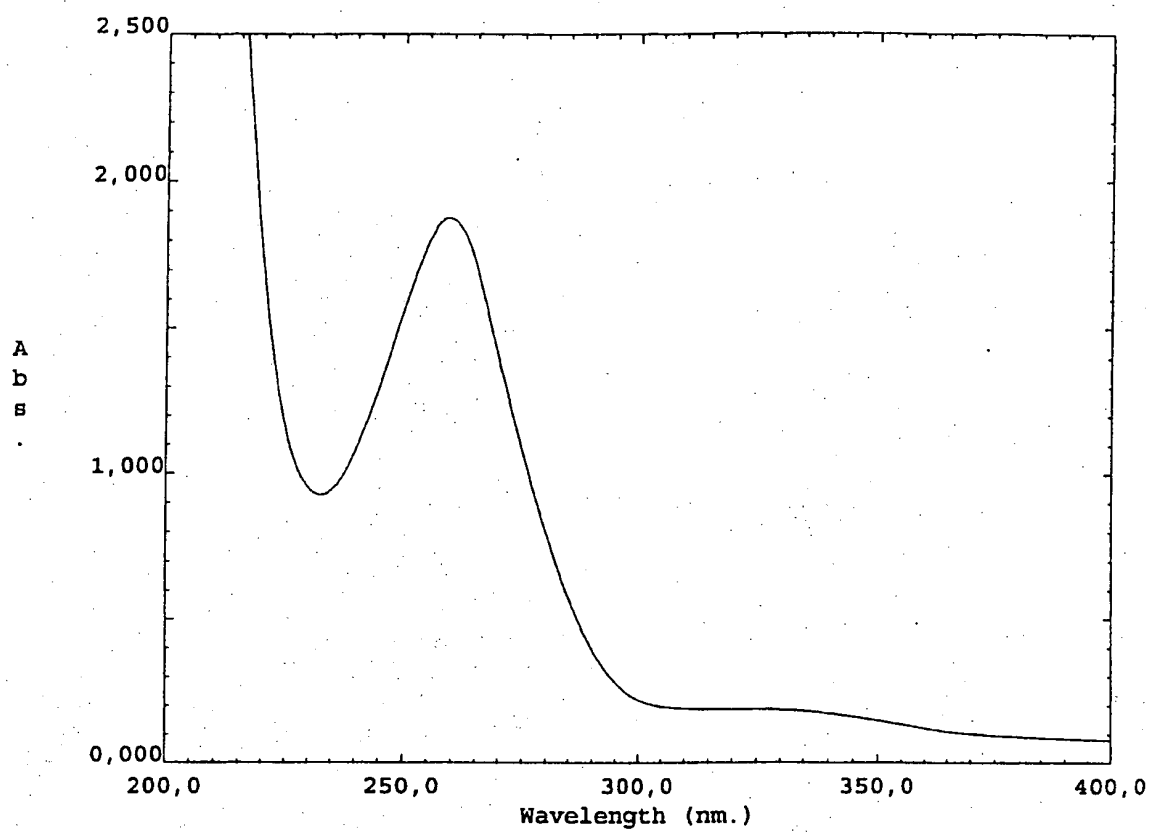


Fig. 11

12/24

Lane  
1 2



Fig. 12

13/24

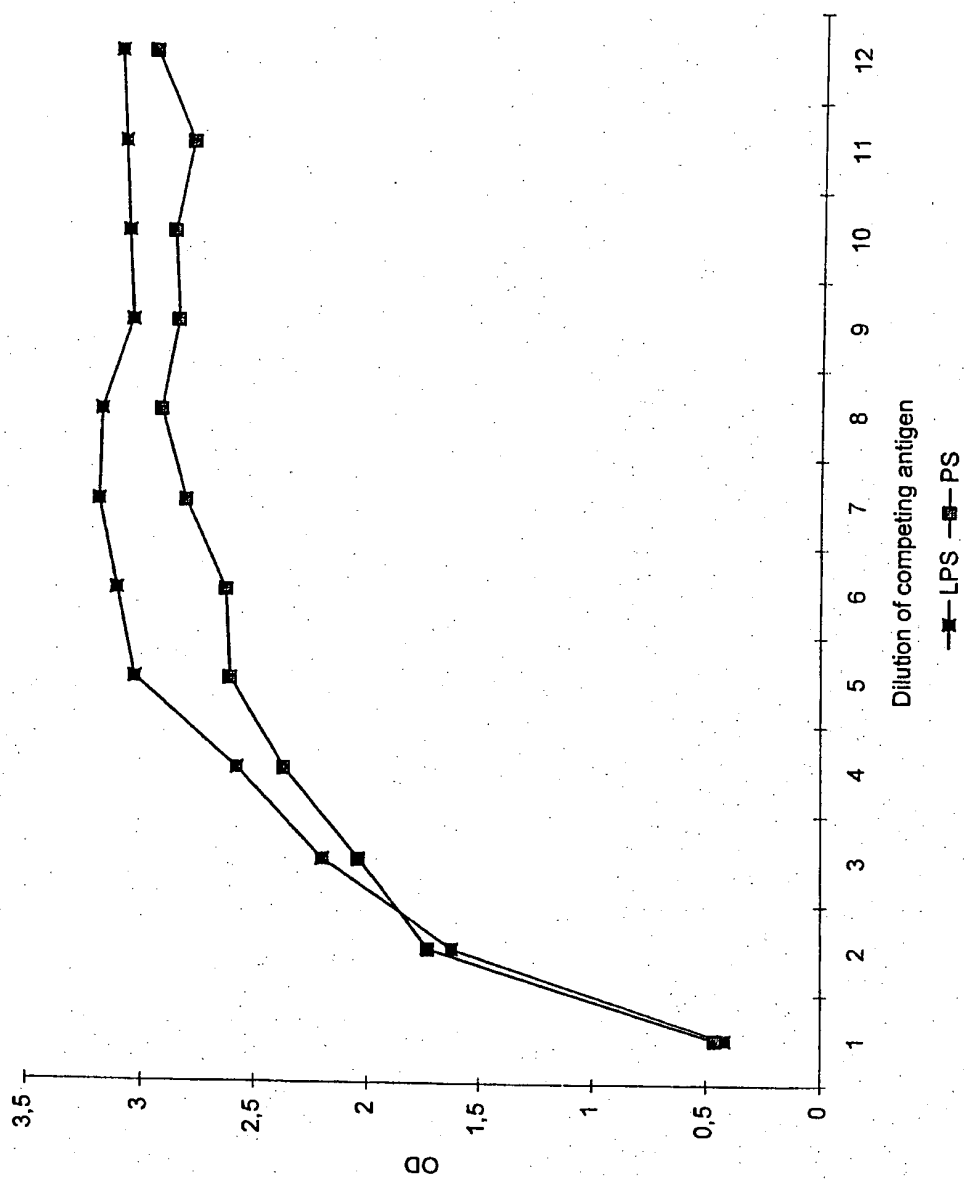


Fig. 13



14/24

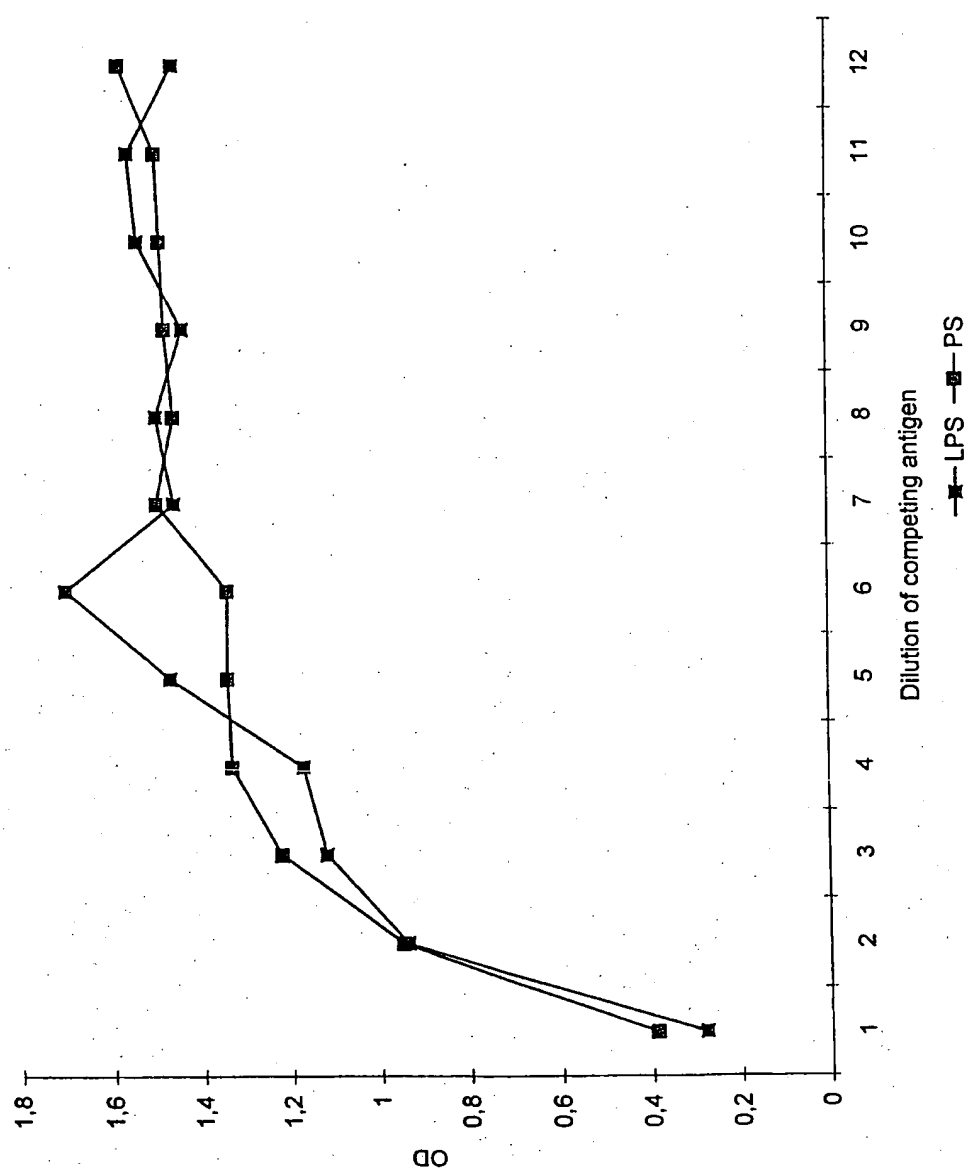


Fig. 14

15/24

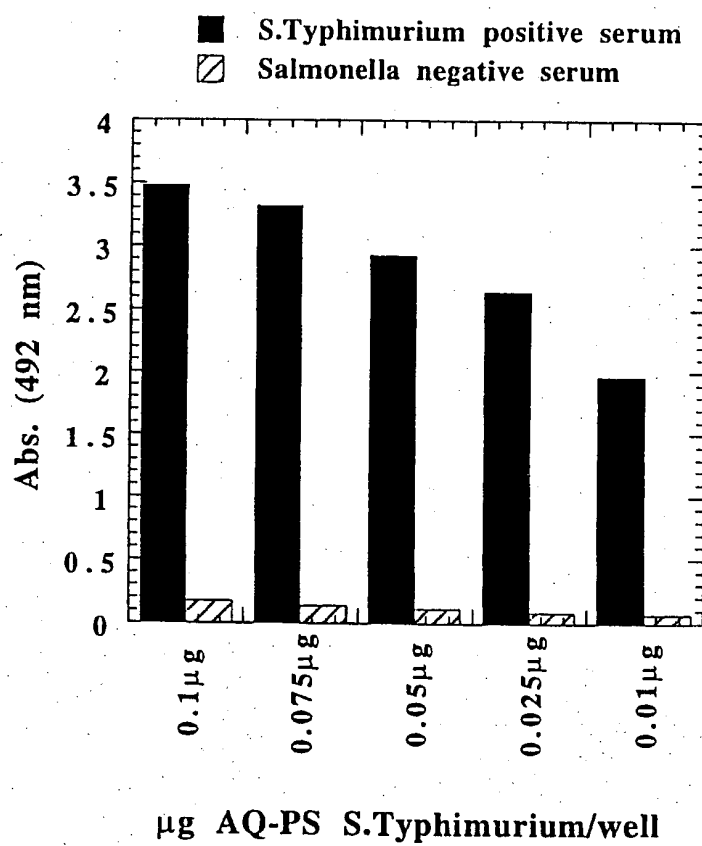


Fig. 15

16/24

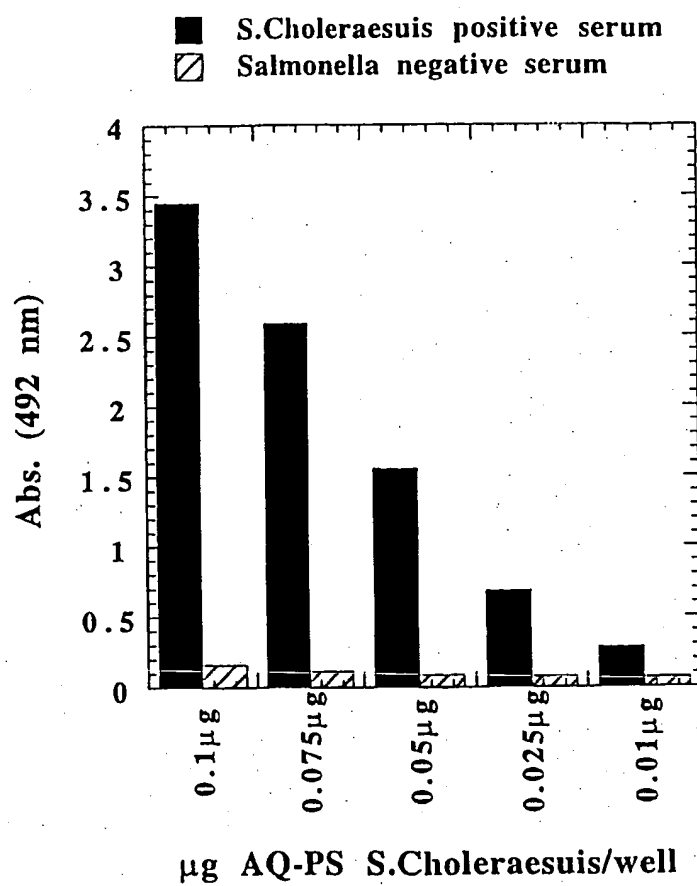


Fig. 16

17/24

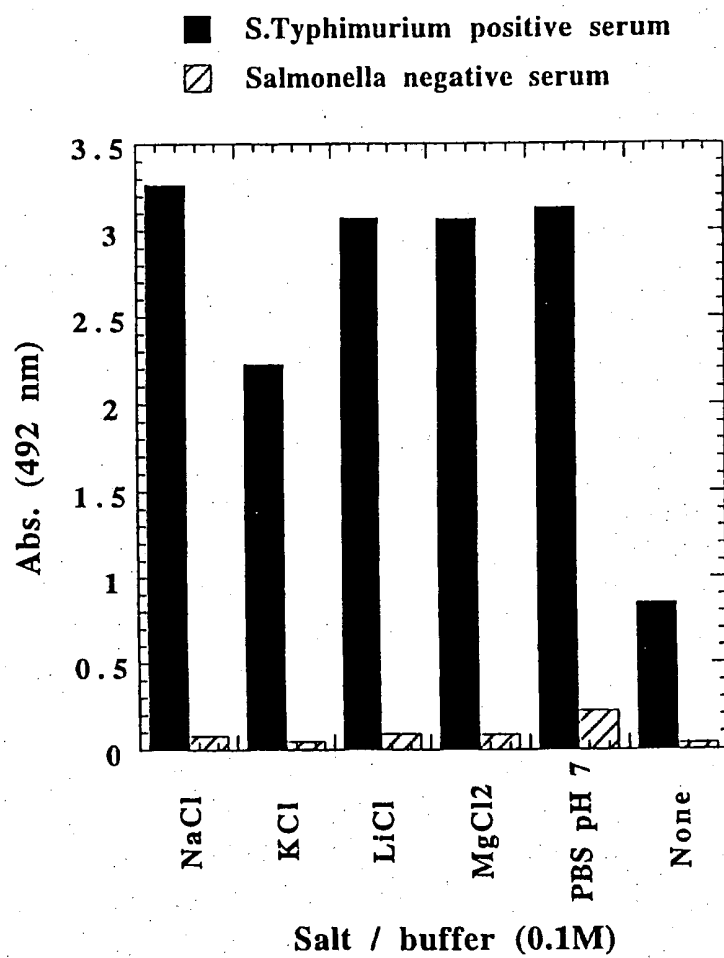


Fig. 17

18/24

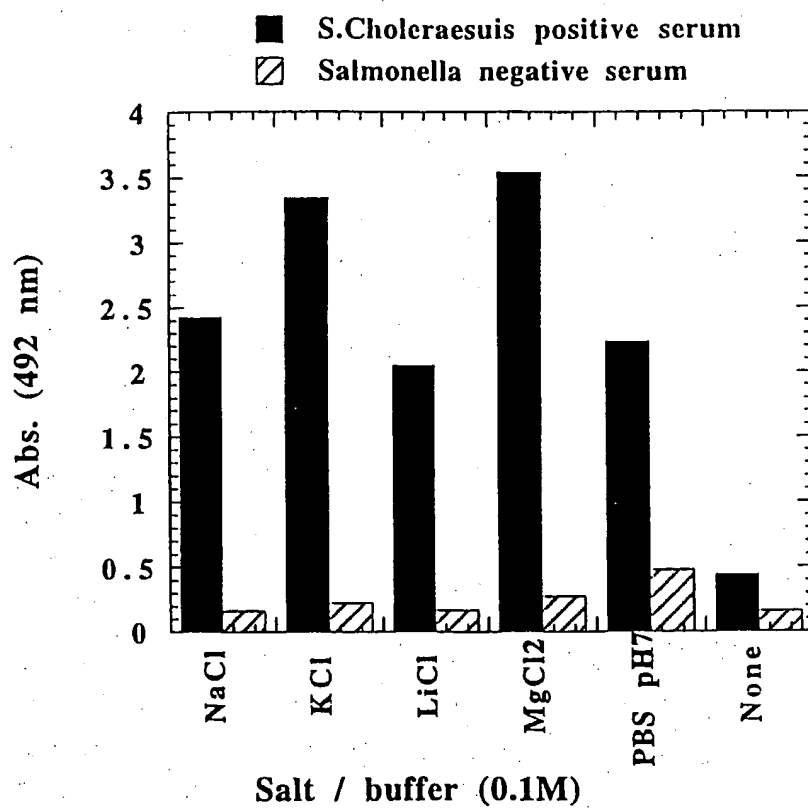


Fig. 18

19/24

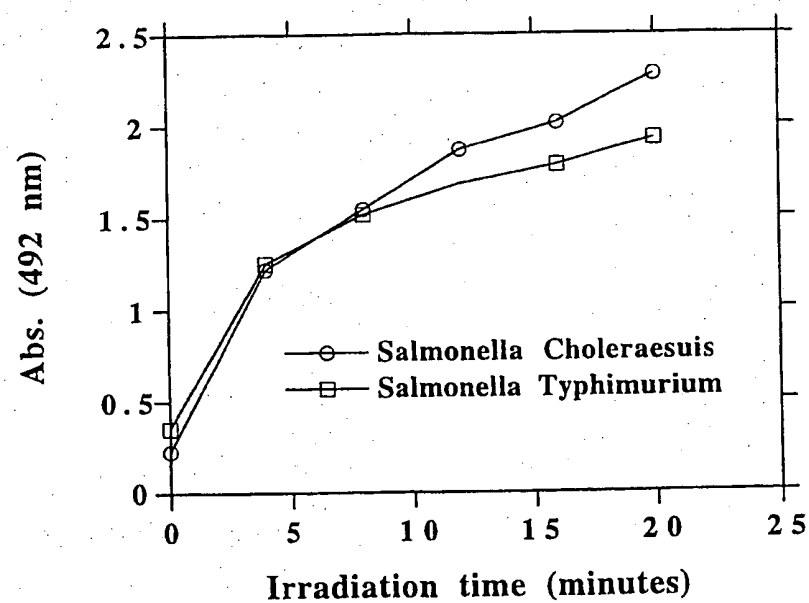


Fig. 19

20/24

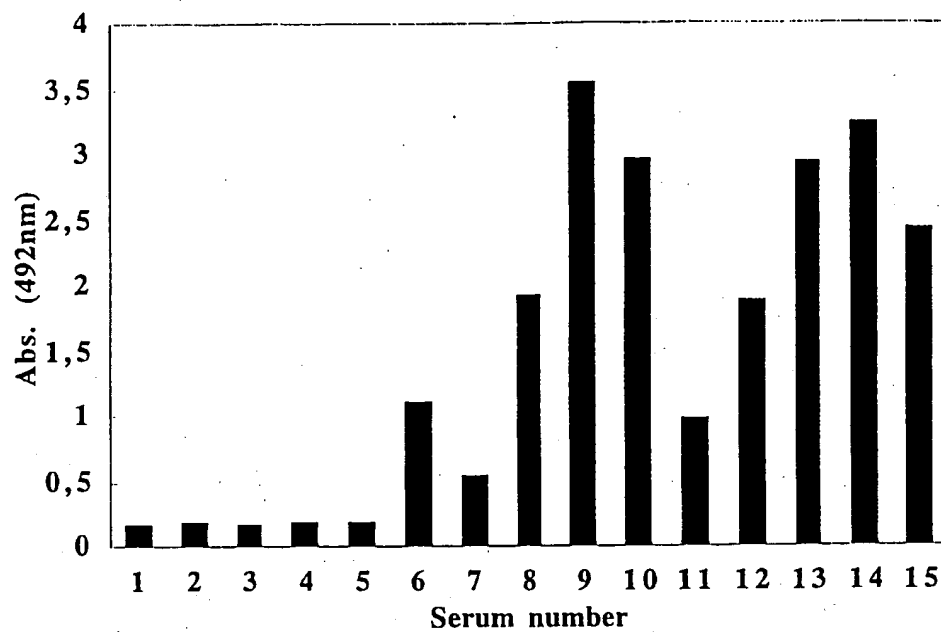


Fig. 20

21/24

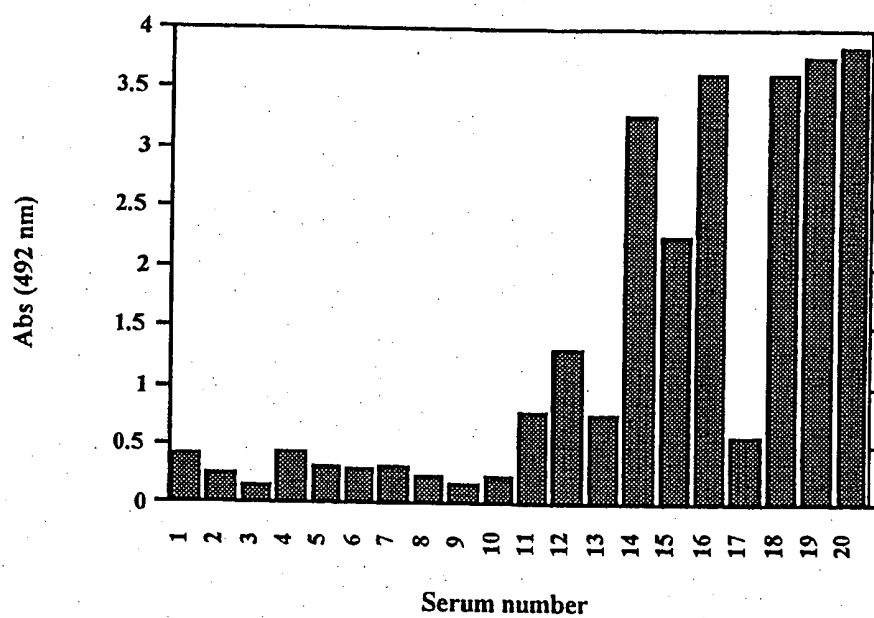


Fig. 21



22/24

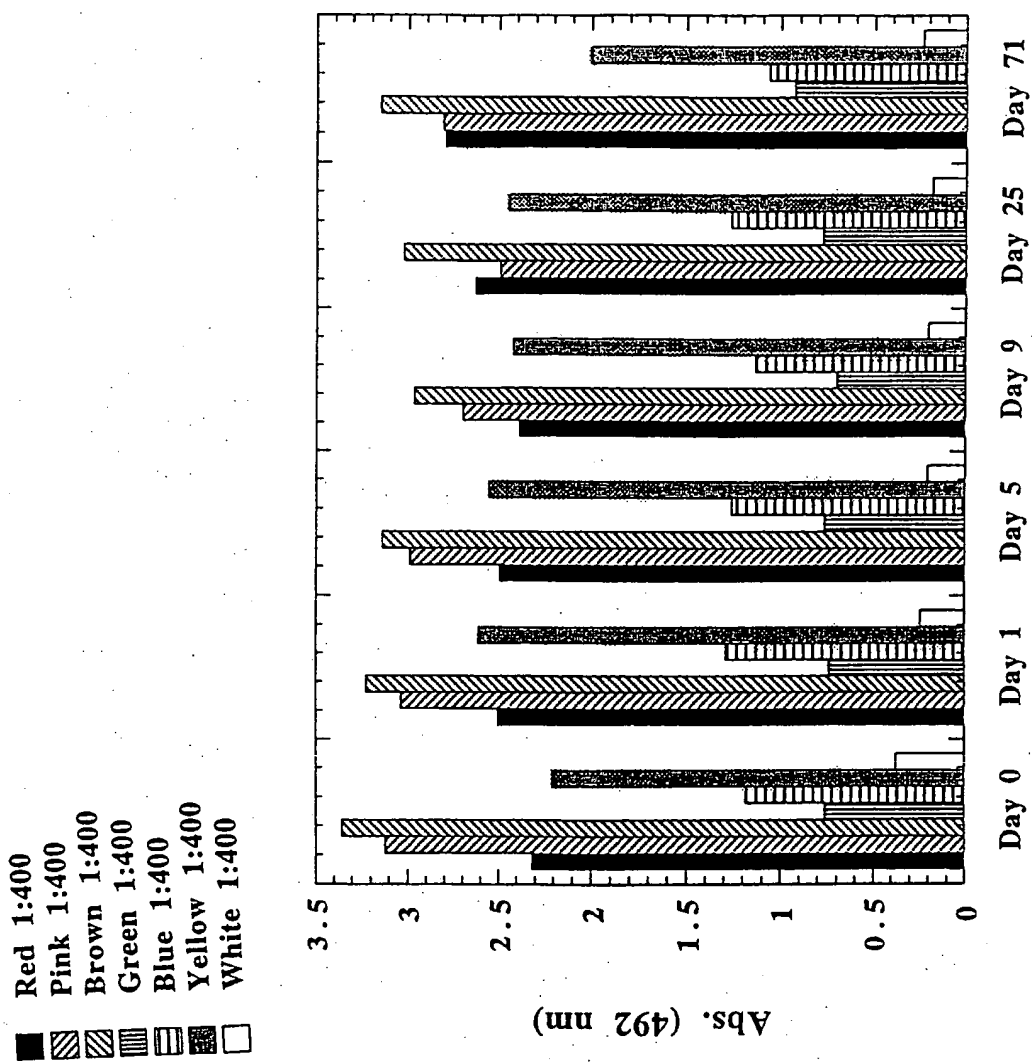


Fig. 22

23/24

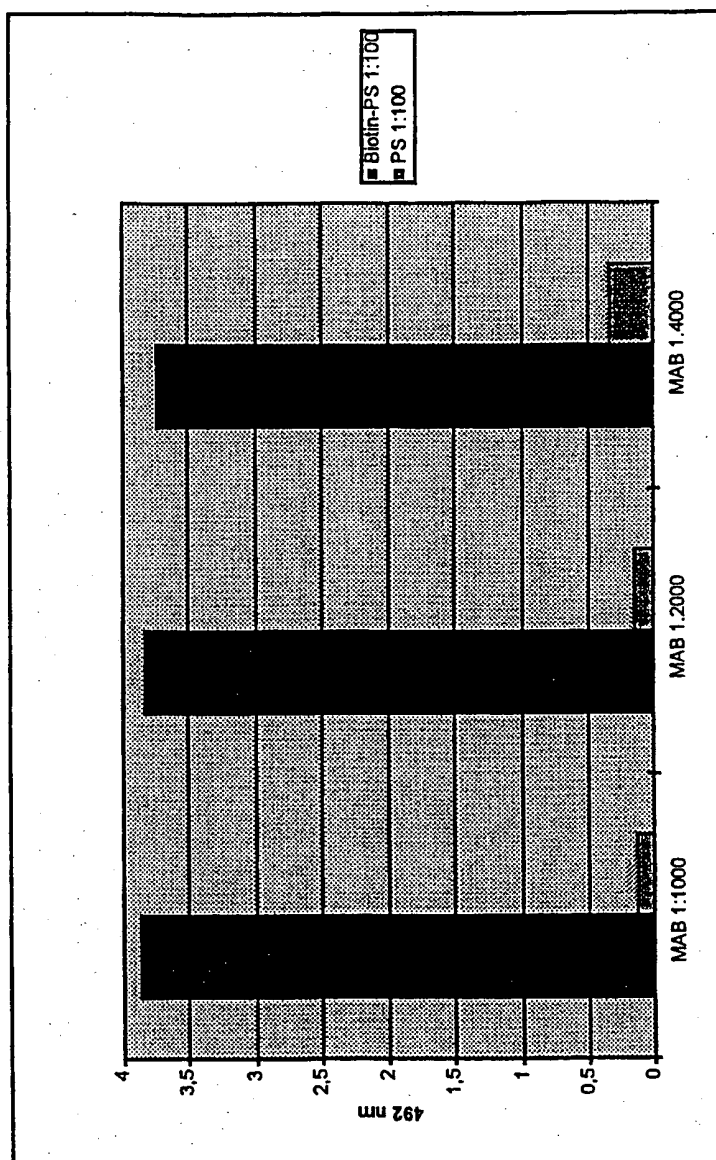


Fig. 23

24/24

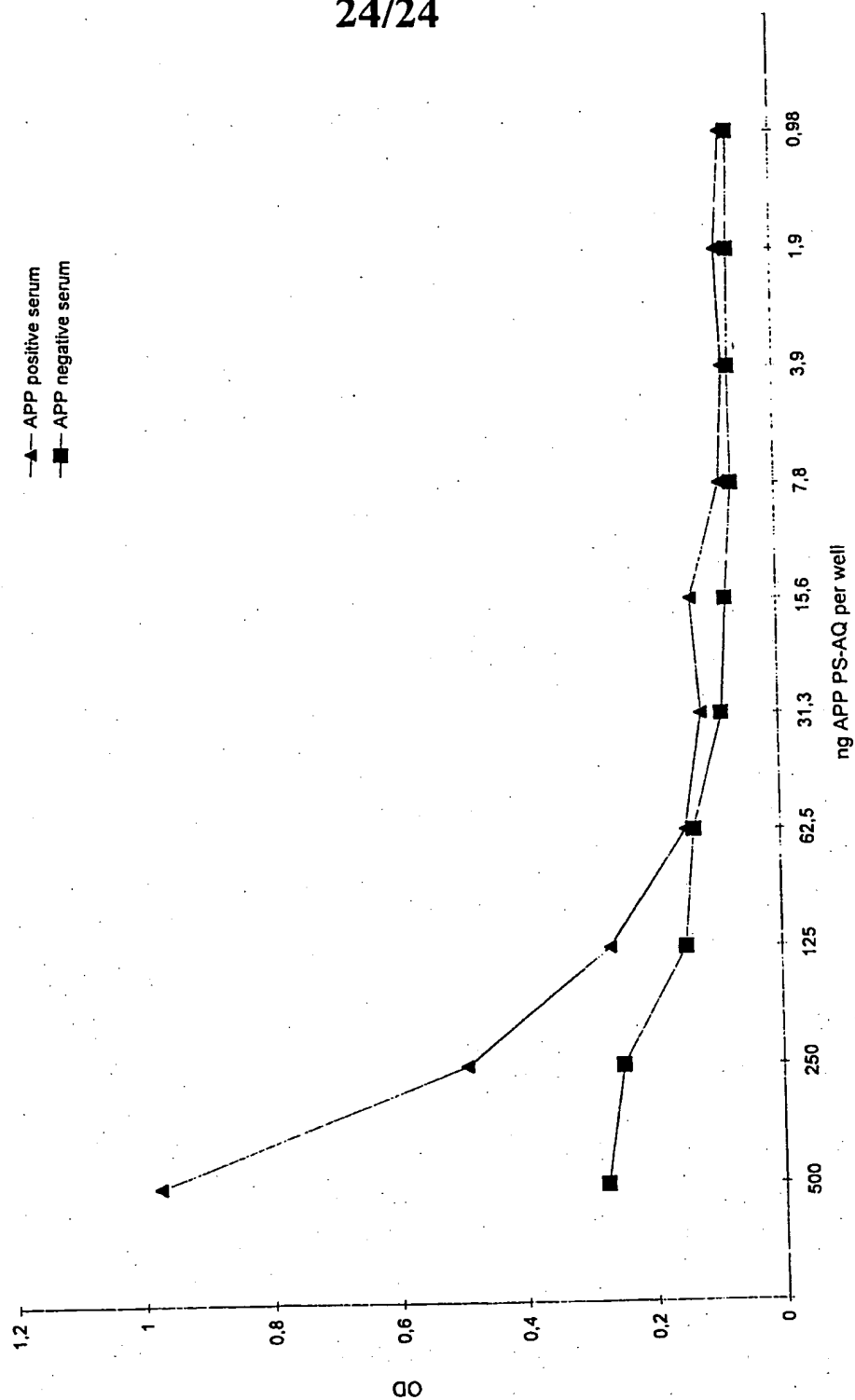


Fig. 24

# INTERNATIONAL SEARCH REPORT

Intern. No. Classification No.

PCT/DK 99/00704

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/569 G01N33/543 C07H3/04 C07H3/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	DE 196 21 488 A (GERLACH GERALD F PROF DR) 4 December 1997 (1997-12-04) the whole document  — -/-	33  31 1,4, 6-10, 21-33

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

3 March 2000

Date of mailing of the international search report

30/03/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Hart-Davis, J

## INTERNATIONAL SEARCH REPORT

Intern. Pat. Classification No.

PCT/DK 99/00704

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	B NIELSEN, D BAGGESEN, F BAGER, J HAUGEGAARD, P LIND: "The serological response to Salmonella serovars typhimurium and infantis in experimentally infected pigs. The time course followed with an indirect anti-LPS ELISA and bacteriological examinations" VETERINARY MICROBIOLOGY, vol. 47, 1995, pages 205-218, XP002103337 cited in the application page 208, paragraph 5 -page 209	33
Y A		31 1,4, 6-10, 21-33
X	EP 0 107 119 A (TORAY INDUSTRIES) 2 May 1984 (1984-05-02) cited in the application example 1	31
A		1-12, 21-23, 26, 34-41, 43-46
X	A ESSWEIN, R BETZ, R R SCHMIDT: "27. Vinyl Carbanions. Part 36. Synthesis of 3-Deoxy-D-manno-2-octulosonic Acid (KDO) and Derivatives" HELVETICA CHIMICA ACTA, vol. 72, 1989, pages 213-223, XP002132147 page 216; figure 3 page 218; figure 4	34,35, 44-46
X	A ESSWEIN, H REMBOLD, R R SCHMIDT: "O-Alkylation at the anomeric centre for the stereoselective synthesis of Kdo-alpha-glycosides" CARBOHYDRATE RESEARCH, vol. 200, 1990, pages 287-305, XP002132148 page 288 page 290 page 291	34,35
Y	PATENT ABSTRACTS OF JAPAN vol. 016, no. 387 (C-0975), 18 August 1992 (1992-08-18) & JP 04 126073 A (BAIO MATERIARU KENKIYUUSHIYO:KK), 27 April 1992 (1992-04-27) abstract	31
A		1-12, 21-23, 26, 34-41, 43-46

-/-

## INTERNATIONAL SEARCH REPORT

Intern. Pat. Classification No.

PCT/DK 99/00704

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	R K GUPTA, W EGAN, D A BRYLA, J B ROBBINS, S C SZU: "Comparative Immunogenicity of Conjugates Composed of Escherichia coli O111 O-Specific Polysaccharide, Prepared by Treatment with Acetic Acid or Hydrazine, Bound to Tetanus Toxoid by Two Synthetic Schemes" INFECTION AND IMMUNITY, vol. 63, no. 8, August 1995 (1995-08), pages 2805-2810, XP002103338 cited in the application page 2805 -page 2806	1-11, 34-41, 44-46
A	A FATTOM, J SHILOACH, D BRYLA, D FITZGERALD, I PASTAN, W W KARAKAWA, J B ROBBINS, R SCHNEERSON: "Comparative Immunogenicity of Conjugates Composed of the Staphylococcus aureus Type 8 Capsular Polysaccharide Bound to Carrier Proteins by Adipic Acid Dihydrazide or N-Succinimidyl-3-(2-Pyridyldithio)propiona te" INFECTION AND IMMUNITY, vol. 60, no. 2, February 1992 (1992-02), pages 584-589, XP002103339 page 587; figure 3	1-11, 34-41, 44-46
A	C GARBAY-JAUREGIBBERY, M C BARSI, A JACQUEMIN-SABLON, J B LE PECQ, B P ROQUES: "Synthesis and Biological Activity of New Dimers in the 7H-Pyrido-'4,3-c!carbazole Antitumor Series" JOURNAL OF MEDICINAL CHEMISTRY, vol. 35, no. 1, 1992, pages 72-81, XP002103340 page 75	1,13-15, 34,44-46
A	WO 96 22534 A (IGEN INC) 25 July 1996 (1996-07-25) page 2, line 3-7, paragraph 2	1,11, 13-17
A	WO 96 31557 A (JACOBSEN MOGENS HAVSTEEN ;KOCH TROELS (DK)) 10 October 1996 (1996-10-10) cited in the application examples 3,4	1,17,18, 21-26

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Publication No

PCT/DK 99/00704

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19621488 A	04-12-1997	AU 3029597 A WO 9745742 A EP 0842431 A	05-01-1998 04-12-1997 20-05-1998
EP 0107119 A	02-05-1984	JP 59211458 A JP 1697652 C JP 3064153 B JP 59064053 A AT 25337 T CA 1204667 A US 5171738 A	30-11-1984 28-09-1992 04-10-1991 11-04-1984 15-02-1987 20-05-1986 15-12-1992
JP 04126073 A	27-04-1992	NONE	
WO 9622534 A	25-07-1996	US 5610075 A AU 4757796 A	11-03-1997 07-08-1996
WO 9631557 A	10-10-1996	AU 699321 B AU 5332996 A CA 2217053 A EP 0820483 A JP 11505554 T	03-12-1998 23-10-1996 10-10-1996 28-01-1998 21-05-1999

THIS PAGE BLANK (USPTO)



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**